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EFFECTS OF THE LIPIDPEROXIDATION PRODUCT 4-HYDROXYNONENAL AND RELATED ALDEHYDES ON PROLIFERATION AND VIABILITY OF CULTURED EHRLICH ASCITES TUMOR CELLS*

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Abstract—The mechanism by which the lipid peroxidation product 4-hydroxynonenal and several other homologous, yet non biogenic aldehydes inhibit proliferation of cultured Ehrlich ascites tumor cells has been studied. Incubation of cells (5×10^{-4}/ml) in a minimum essential medium supplemented with 10 or 20 μM 4-hydroxynonenal reduces the 36-hr cell count to 65 and 30% of the control value. The reduced growth rate is most likely due to a blockage of the DNA synthesis. Cells labelled by a [^{3}H]-thymidine pulse prior to exposure to 4-hydroxynonenal (20 μM, 8 hr) showed no change of the specific radioactivity of the DNA, indicating that no *de novo* synthesis occured in the presence of the aldehyde. In the absence of the aldehyde the specific radioactivity of the DNA decreased by 25%. A 2-hr incubation in the presence of 10 or 20 μM of 4-hydroxynonenal reduced [^{3}H]-thymidine incorporation into the $HClO_4$ insoluble fraction to 85 and 50% of the controls, but had no effect of the [^{3}H]-thymidine and ^{86}Rb uptake. Moreover, examination of the cell cultures by the Trypan Blue exclusion technique revealed that 20 μM 4-hydroxynonal does not cause cell death. The high reactivity of 4-hydroxynonenal towards sulfhydryl groups suggests that the aldehyde inhibits DNA synthesis by interacting with a functional SH group of DNA polymerase. The specific action on DNA synthesis is abolished at an aldehyde concentration of 50 μM, which leads to 30% (6 hr exposure) and 95% (36 hr exposure) of dead cells.

The cytostatic index (CI), i.e. concentration at 50% Trypan Blue positive cells/concentration at 50% inhibition of cell growth deducted from the dose effect curves is 3.0 for 4-hydroxynonenal. The other homologous 4-hydroxyalkenals with chain length of 5, 6, 7, 8, 10 and 11 carbon atoms also inhibit cell growth. The CI varied from 1.20 to 1.94, indicating that these non biogenic 4-hydroxyalkenals have a distinctively lower specific effect on proliferation than the biogenic 4-hydroxynonenal.

The Michael adducts of 4-hydroxynonenal with glutathione and cysteine were nearly one order of magnitude less toxic than the free aldehyde, the CI (2.41 cysteine adduct, 2.06 glutathione adduct), however, were not improved since the growth inhibitory action was also reduced.

The 4-hydroxyalkenals were discovered many years ago as autoxidation products of polyunsaturated fatty acids [1, 2]. Later on it was shown that such aldehydes, in particular 4-hydroxynonenal, are also formed in biological specimens, such as liver microsomes and liver cells upon exposure to pro-oxidant stimuli [3, 4]. Very recently 4-hydroxynonenal was detected as one of the toxic components in oil samples from the toxic oil syndrome outbreak in Spain [5]. 4-Hydroxyalkenals are chemically very reactive and show a diverse spectrum of biological effects. They inhibit growth of certain transplanted animal tumors [6] and are mutagenic in selected *Salmonella* tester strains [7]. The biogenic aldehyde 4-hydroxynonenal affects the plasma membrane adenylate cyclase at concentrations of 1 μM and below [8] and exerts chemotactic and chemokinetic effects towards polymorphonuclear leucocytes [9]. In clinical tests aqueous solutions of 4-hydroxypentenal were successfully used in the treatment of human basaliomata [10] and cervical carcinoma [11]. Many other effects of 4-hydroxypentenal and related aldehydes on different cells and cell fractions and isolated enzymes have been reported [12–14]. At neutral pH 4-hydroxyalkenals react preferentially with SH groups of both proteins and non proteins and it has been concluded that most, of not all, biological effects are due to the blockage of functional SH groups [6, 15]. It should be noted, that these aldehydes are not merely unspecific SH blockers but exhibit high selectivity and can, at least at low concentrations, discriminate between different types of thiols in the cell. At concentrations leading to a significant reduction of the incorporation of [^{3}H]-thymidine, 4-hydroxyalkenals mainly react with nuclear SH groups, while the intracellular glutathione and cytoplasmic SH-proteins are not or much less affected [16]. The inhibition of incorporation of labelled precursors into the trichloroacetic acid insoluble fraction brought about by 4-hydroxyalkenals has been interpreted as an inhibition of DNA synthesis [17, 18]. It should be considered, however, that reduced incorporation of precursors into the acid insoluble fraction can also be

* Dedicated to Professor Karl Decker (Freiburg) on his 60th birthday.

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produced by several other mechanisms and is not necessarily the result of an inhibition of DNA synthesis [19]. Therefore, we studied the effects of 4-hydroxyalkenals on the proliferation and DNA synthesis in cultured Ehrlich ascites tumor cells using additional procedures. The results strongly suggest, that 4-hydroxyalkenals, in particular the biogenic aldehyde 4-hydroxynonenal, inhibit growth of Ehrlich ascites tumor cells by blockage of DNA synthesis.

MATERIALS AND METHODS

Chemicals (cysteine, glutathione, buffers) were purchased from E. Merck (Darmstadt); minimal essential medium (MEM) and calf serum were obtained from Eurobio (Paris); penicillin, streptomycin sulfat and fine chemicals were delivered from Serva (Heidelberg), the radiochemicals ([^{3}H]-thymidine and ^{86}-Rb) were purchased from Radiochemical Center, Amersham.

4-Hydroxyalkenals were synthesized as described [20]. The 4-hydroxynonenal–cysteine adduct was prepared essentially as described for the 4-hydroxypentenal–cysteine adduct [25]. 100 ml aqueous cysteine solution were added dropwise under stirring to an aqueous solution containing 8.611 g (= 55.2 mmoles) 4-hydroxynonenal. The cysteine solution contained 6.688 g cysteine zwitterion (55.2 mmoles) and the pH of the solution was 4.7. After addition of cysteine the pH of the solution was adjusted to 7.0 with diluted NaOH. The solution was kept at ambient temperature for 4 hr. In this time period the free hydroxynonenal concentration decreased to 0.1% of the original value. The solution was concentrated on a rotary evaporator (30°) to 200 ml. The concentrate was extracted twice with 50 ml chloroform and the aqueous phase was lyophilized. The product obtained was a white, dry powder. The yield was 9.3 g. The product was stored until use at 4°. The preparation of the 4-hydroxynonenal–glutathione adduct followed essentially the conditions used for the kinetic studies of the reaction mechanism [21]. Specifically, 300 mg 4-hydroxynonenal dissolved in 70 ml water were mixed with 594 mg glutathione dissolved in 100 ml water. The pH of the mixture was adjusted to 7.8 with diluted NaOH. After 1 hr at room temperature the concentration of the free aldehyde decreased to 0.025% of the original value. The solution was extracted once with 50 ml chloroform and the aqueous phase was lyophilized. The product obtained (800 mg) was a dry, white powder and was stored until use at 4°.

Glycogen positive Ehrlich ascites tumor cells were cultured in minimal essential medium with penicillin and streptomycin (100 μg/ml medium), 4% calf serum, 2% new-born calf serum; the doubling time of Ehrlich ascites tumor cells in culture in logarithmic phase is 19 hr. The cells were harvested in the logarithmic period and diluted with medium to a cell number of 1–5 $\times$ 10^4 cells/ml. After 1 hr pre-incubation, the 4-hydroxyalkenals or the thiol-adducts were added to the corresponding cell cultures as indicated. At this time an aliquot was tested for viability using Trypan Blue exclusion. After 6 and 36 or 45 hr the cells were checked again for cell number and viability as indicated. [^{3}H]-thymidine incorporation was measured in the acid (0.7N $HClO_4$) insoluble pellet. DNA in this pellet was determined by a modification of the Ceriotti procedure as described by Keck [22] using calf thymus DNA as a standard.

For thymidine uptake the cells were preincubated in the presence of various concentrations of 4-hydroxynonenal for 4 hr; [^{3}H]-thymidine was added for 20 min and then the radioactivity contained in the cells was estimated by rapid centrifugation through silicone oil [23]. All individual values were related to the control values (= 100%) obtained from cultures where the aldehyde was omitted. The values given are average of three independent experiments. For measurement of ^{86}Rb uptake, the cell cultures were pre-incubated with 1–5 $\times$ 10^{-5} M 4-hydroxynonenal for 4 hr (control without the aldehyde). ^{86}Rb was added to each batch and 45 and 90 sec thereafter the amount of ^{86}Rb taken up by the cells was determined by the silicon filtration method [23]. All values were related to the control samples (= 100%) where 4-hydroxynonenal was omitted.

RESULTS

(1) *Effects of 4-hydroxynonenal*

The dose dependence (2 $\times$ 10^{-6}–5 $\times$ 10^{-4} M) of the effect of 4-hydroxynonenal on proliferation, thymidine incorporation, thymidine uptake and cell viability of cultured Ehrlich ascites tumor cells was studied (Fig. 1). At concentrations of 5 $\times$ 10^{-6} M

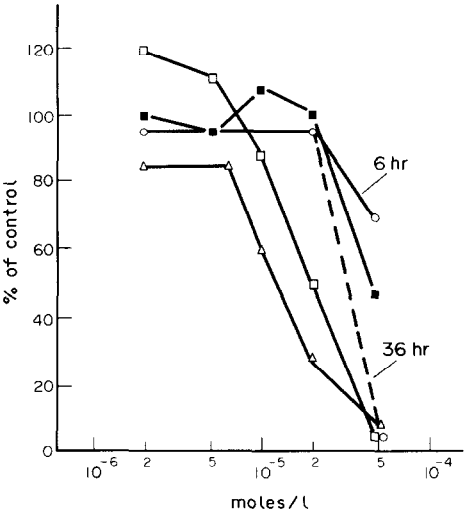

Fig. 1. Effect of 4-hydroxynonenal on cell growth (△–△), viability (○–○) 6 hr, (○· · ·○) 36 hr, [^{3}H]-thymidine incorporation (□–□), and [^{3}H]-thymidine uptake (■–■) in Ehrlich ascites tumor cell cultures. For the determination of the effects on cell growth the cells were incubated for 36 hr in the presence of 4-hydroxynonenal; for viability 6 hr and 36 hr as indicated; for thymidine incorporation 2 hr, and for thymidine uptake 4 hr. Coefficient of variation: for cell number (cell growth) ± 4.8%, for Trypan Blue exclusion (viability) ± 1.9%, for thymidine incorporation ± 6.2%, and for thymidine uptake ± 10% (in controls 4-hydroxynonenal was omitted).

and below the aldehyde has no effect on these functions only a weak stimulation of [^{3}H]-thymidine incorporation is observed (Fig. 1). With increasing concentrations cell proliferation is reduced to 65% (1×10^{-5} M), 30% (2×10^{-5} M), 7% (5×10^{-4} M) of the control value observed in the absence of the aldehyde. Parallel to the inhibition of cell growth, although at somewhat higher concentrations, 4-hydroxynonenal also blocks the incorporation of [^{3}H]-labelled thymidine into the DNA, i.e. $HClO_4$ insoluble fraction. The other two parameters studied i.e. thymidine uptake and cell viability, are much less sensitive towards the aldehyde. A concentration of 2×10^{-5} M 4-hydroxynonenal which leads to a 70% inhibition of cell growth and 50% inhibition of thymidine incorporation has no measureable effect on the thymidine uptake or on the cell viability. These parameters are only affected if the concentration is increased to 5×10^{-5} M.

To prove or disprove whether the reduced incorporation of labelled thymidine into DNA results from the inhibition of *de novo* DNA synthesis or from an inhibition of the thymidine uptake, DNA pulse label experiments were performed. Specifically, Ehrlich ascites tumor cell cultures were incubated with [^{3}H]-thymidine for 3 days. Thereafter the cells were harvested, washed, and incubated in [^{3}H]-thymidine free medium for 24 hr. After this time period no acid soluble radioactivity was measureable indicating that all the free thymidine from the pulse label has been removed. The pulse prelabelled cells were then incubated in the absence (control) and presence (2×10^{-5} M) of 4-hydroxynonenal. After 8 hr the specific radioactivity of the DNA compared to the non incubated control was estimated by measuring both the total amount of DNA and the radioactivity. Table 1 shows that in cells incubated in the absence of 4-hydroxynonenal the specific radioactivity of the pulse labelled DNA decreased by about 25% due to the newly synthesized DNA, whereas the specific radioactivity remained unchanged in the cells exposed to 2×10^{-5} M 4-hydroxynonenal. This clearly demonstrates that the aldehyde in fact blocks the DNA synthesis. These pulse label experiments strongly suggest that inhibition of the incorporation of [^{3}H]-thymidine into the $HClO_4$ insoluble fraction (Fig. 1) reflects actually the inhibition of the incorporation of thymidine into the DNA.

This conclusion is further confirmed by the [^{3}H]-thymidine and ^{86}Rb uptake experiments. The concentration of 2×10^{-5} M 4-hydroxynonenal has virtually no inhibitory action on uptake of thymidine by Ehrlich ascites tumor cells (Fig. 1). The uptake of ^{86}Rb (Table 2), as a further membrane associated process (indicating the $Na^+/K^+/Cl^-$ cotransport system and the K^+/Na^+ dependent ATPase), is also not significantly reduced as compared to the control. Concentrations of 5×10^{-5} M are needed to obtain approximately 50% inhibition of these processes. It is worth noting, that the dose dependence of the reduction of viability by 4-hydroxynonenal apparently coincides with the inhibition of thymidine and rubidium uptake which are both membrane associated processes. Therefore, it seems plausible to assume that the cell death caused by 5×10^{-5} M 4-hydroxynonenal is in some way related to damaging effects on the cell membrane.

Table 1. Effect of 2×10^{-5} M 4-hydroxynonenal on the specific radioactivity of pulse labelled DNA in Ehrlich ascites tumor cell cultures

		dpm/mg DNA
Control	after 0 hr	6360 ± 460
	after 8 hr	4662 ± 370
2×10^{-5} HNE	after 8 hr	6456 ± 1270

(Values of 10 determinations ± S.E.M.); HNE = 4-hydroxynonenal.

Table 2. Effect of 4-hydroxynonenal on ^{86}Rb uptake in Ehrlich ascites tumor cell cultures

		^{86}Rb uptake in % of the control
Control		100
4-hydroxynonenal	5×10^{-6} M	110
	2×10^{-5} M	90
	5×10^{-5} M	51

Cultures of Ehrlich ascites cells were preincubated in the presence and absence (= control) of 4-hydroxynonenal for 4 hr, ^{86}Rb was then added; 45 and 90 sec later the ^{86}Rb uptake by the cells was determined by the silicone filtration method [23]. The values are the means of the two time points.

(2) *Effects of 4-hydroxypentenal*

4-Hydroxypentenal is a homologue to 4-hydroxynonenal, it has the same functional group (—CHOH—CH=CH—CHO) and has the same chemical reactivity. 4-Hydroxypentenal due to the lack of CH_2 groups in the tail, is a less lipophilic compound. Based on the distribution coefficient between water and chloroform (4-hydroxypentenal: 4.5; 4-hydroxynonenal: 0.04) the lipophilicity of 4-hydroxypentenal lies about two orders of magnitude below that of 4-hydroxynonenal [14]. The effects of drugs are often strongly influenced by the lipophilic and/or hydrophobic character, therefore, a quantitative comparison of the effects of 4-hydroxypentenal and 4-hydroxynonenal on Ehrlich ascites tumor cells could contribute to the design of new and more effective drugs with the same biological active group. The dose dependent effects of 4-hydroxypentenal on proliferation, [^{3}H]-thymidine incorporation, and viability of cultured Ehrlich ascites tumor cells were investigated in the concentration range of 2×10^{-6} to 5×10^{-4} M (Fig. 2). Concentrations of 2×10^{-5} M and below had no significant effect on these parameters. Compared on a molar base 4-hydroxypentenal is a less powerful inhibitor of cell proliferation and thymidine incorporation than 4-hydroxynonenal, which leads to strong reduction of these two processes at 2×10^{-5} M. With increasing concentrations 4-

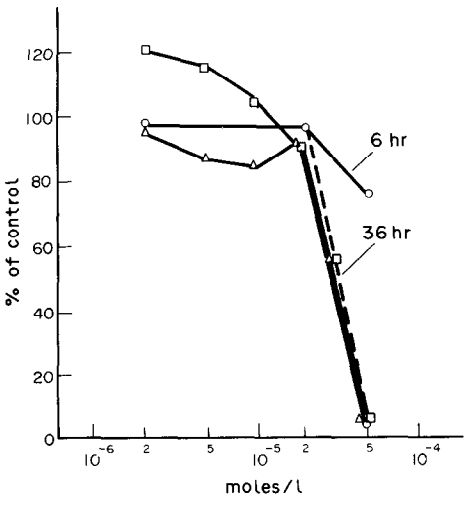

Fig. 2. Effect of 4-hydroxypentenal on cell growth (△–△), viability (○–○) 6 hr, (○· · ·○) 36 hr, and [^{3}H]-thymidine incorporation (□–□). The cells were incubated in the presence of 4-hydroxypentenal as indicated in Fig. 1 for 4-hydroxynonenal.

hydroxypentenal reduced cell proliferation, thymidine incorporation and viability to 50% (3×10^{-5} M) and 5% (5×10^{-5} M) of the control value. There are no significant differences in the sensitivity of these processes towards the aldehydes, as was observed in the case of hydroxynonenal. The inhibitory action of 4-hydroxypentenal on proliferation and DNA synthesis which might exist, is masked by the general unspecific cytotoxicity of the drug. Therefore, it is not possible to discriminate between these effects with certainty. The comparison of the dose–effect relationship of 4-hydroxypentenal and 4-hydroxynonenal (Figs. 1 and 2, and Table 3) clearly shows that the lipophilicity of a hydroxyalkenal is an important factor in determining the selective action on certain cell processes. Compared to 4-hydroxypentenal, the more lipophilic 4-hydroxynonenal inhibits proliferation already at concentrations where it does not affect cell viability.

These considerations suggest that 4-hydroxyalkenals with longer chain length would even give a more pronounced and selective inhibition of cell proliferation. To test this hypothesis a series of 4-hydroxyalkenals differing in chain length were investigated.

(3) *Relationship between chain length of 4-hydroxyalkenals and their effects on cell growth and cell viability*

Seven 4-hydroxyalkenals with chain length from five carbon atoms (4-hydroxypentenal) to 11 carbon atoms (4-hydroxyundecenal) were tested in the Ehrlich ascites tumor cell model system in respect to their dose dependent effects on cell growth and cell viability. The first parameter is taken as a measure for a selective and specific effect of the aldehyde on the tumor cells while the second parameter is an index for the "nonspecific" cytotoxic effects of the drugs. From the dose–effect curves (Figs. 1 and 2) the concentrations giving in a 45 hr incubation period 50% growth inhibition and 50% Trypan Blue positive cells were estimated.

The ratio between concentrations giving 50% Trypan Blue positive cells and concentrations giving 50% growth inhibition we call the cytostatic index CI. The CI is a practical parameter for the quantitative comparison of the selective proliferation inhibitory activity of different drugs. Table 3 lists the cytotoxicity, growth inhibitory activity and the CI for the seven investigated 4-hydroxyalkenals. It can be seen that in the series investigated 4-hydroxynonenal has the highest CI (CI = 3.0), whereas all other aldehydes have indices in the range of 1.20 (hydroxyhexenal) to 1.94 (hydroxydecenal).

The preliminary hypothesis deduced from the comparison between 4-hydroxypentenal and 4-hydroxynonenal, that the CI increases with increasing lipophilicity of the aldehyde, i.e. with increasing chain length, is therefore not verified by these con-

Table 3. Effect of 4-hydroxyalkenals with chain length from 5 to 11 carbon atoms and Michael adducts of 4-hydroxynonenal on the growth and viability of Ehrlich ascites tumor cells

Chain-length	50% inhibition of cell growth (moles/l)	50% loss of viability (moles/l)	CI
C_5	1.40×10^{-5}	2.45×10^{-5}	1.75
C_6	2.05×10^{-5}	2.45×10^{-5}	1.20
C_7	1.15×10^{-5}	1.60×10^{-5}	1.39
C_8	1.05×10^{-5}	1.40×10^{-5}	1.33
C_9	7.50×10^{-6}	2.25×10^{-5}	3.0
C_{10}	8.25×10^{-6}	1.60×10^{-5}	1.94
C_{11}	1.10×10^{-5}	1.70×10^{-5}	1.54
HNE-Cysteine	5.40×10^{-5}	1.30×10^{-4}	2.41
HNE-Glutathione	6.80×10^{-5}	1.40×10^{-4}	2.06
N-Lost HN2	2×10^{-6}	2×10^{-4}	100

The values were determined from the dose effect curves measured 45 hr after addition to the aldehydes; cytostatic index (CI): Concentration at 50% Trypan Blue positive cells/concentration at 50% inhibition of cell growth; HNE = 4-hydroxynonenal.

tinued studies and must be rejected. The reason why 4-hydroxynonenal is clearly superior to the other 4-hydroxyalkenals is not clear. It might be that both, cytotoxicity and growth inhibition of 4-hydroxyalkenals, are strongly influenced by the capacity of the cell to detoxify the aldehyde. It is known from other studies [24], that the rate of metabolism of 4-hydroxyalkenals by isolated hepatocytes increases nearly linearly with chain length. Assuming a similar situation for the Ehrlich ascites tumor cells the expected increase of growth inhibition with chain length would be abolished at least in part by the increased metabolic inactivation. The observed maximum of the CI for the 4-hydroxynonenal would then result from the superimposition of the cytostatic and growth inhibitory capacity with the metabolic inactivation of the aldehyde, increasing with increasing chain length. Besides this possible explanation one should also keep in mind that the peculiar selectivity of 4-hydroxynonenal might result from the fact that this is actually a biogenic compound.

Compared to 2-chloro-*N*-(3-chloroethyl)-*N*-methylenthanamine (HN2) which has a CI of 100 (see Table 3), the 4-hydroxynonenal is a much less specific inhibitor of Ehrlich ascites tumor cell growth. Nevertheless, the fact that 4-hydroxynonenal can be formed intracellularly from membrane lipids by a radical induced lipid peroxidation [3, 4] and that the aldehyde can be converted into many other masked and possibly less toxic forms [6] justifies the anticipation that 4-hydroxynonenal derivatives can be synthesized exhibiting lower non specific cytotoxicity without loss of cytostatic activity.

(4) *Effect of some 4-hydroxynonenal derivatives on cell growth and cell viability*

Due to the high reactivity of 4-hydroxynonenal towards thiol groups, derivatives containing the aldehyde bound to cysteine, cysteamine, glutathione, SH-proteins and many other SH compounds can easily be prepared [15, 21, 25] according to the equation: RSH+R'CHOH—CH=CH—CHO⇄ R'—CHOH—CH(SR)—CH_2—CHO. All these compounds contain therefore the aldehyde in a masked form as Michael adducts (i.e. saturated aldehydes with the thiol bound by a thioether linkage at carbon 3). The Michael adducts can release the aldehyde again in the reverse reaction provided that it is scavenged by another thiol having a higher affinity to the aldehyde than the parent thiol RSH. It was shown that such aldehyde–thiol adducts [26–28] (most intensively studied were those of crotonaldehyde and hydroxypentenal) are much less toxic for mice and rats as compared to the free aldehyde and that such adducts can inhibit or prevent growth of Ehrlich ascites tumor cells in mice. It was also shown that some aldehyde–thiol adducts inhibit incorporation of [^{3}H]-thymidine into the acid insoluble fraction of Ehrlich ascites tumor cells [6]. With this background we studied the Michael addition compounds of 4-hydroxynonenal with cysteine and with glutathione. Figure 3 shows the dose–effect curve of the 4-hydroxynonenal–cysteine adduct in comparison to the free aldehyde. The binding of the aldehyde to cysteine significantly reduces the cytotoxicity. The free aldehyde gives 50% Trypan

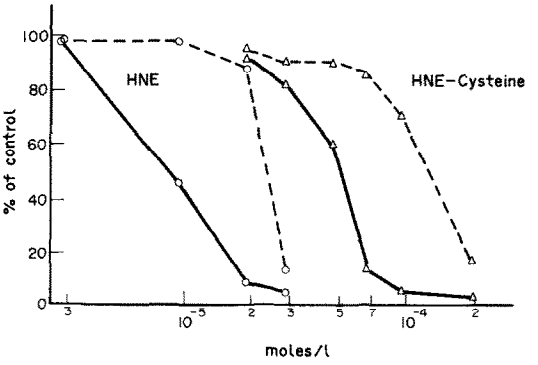

Fig. 3. Effect of 4-hydroxynonenal and 4-hydroxynonenal-cysteine adduct on cell growth and viability. The cells were incubated for 45 hr in the presence of the drugs. 4-Hydroxynonenal: cell growth (O–O), viability (O· · ·O); 4-hydroxynonenal-cysteine adduct: cell growth (△–△), viability (△· · ·△); HNE = 4-hydroxynonenal.

Blue positive cells at 2.4×10^{-5} M, in the case of using the cysteine adduct about 1.3×10^{-4} M are needed. But the growth inhibition curve is also shifted to higher concentrations and a concentration of 5.4×10^{-5} M of the cysteine adduct is necessary to reduce cell growth by 50%. The CI for this compound is 2.41 and therefore not improved as compared to the parent aldehyde. A similar situation exists for the 4-hydroxynonenal–glutathione adduct (Fig. 4) which has an CI of 2.06.

DISCUSSION

Cultured Ehrlich ascites tumor cells exposed to concentrations of 10–20 μM 4-hydroxynonenal respond with a strong inhibition of the proliferation. These concentrations of the aldehyde do not affect cell viability and membrane integrity measured by the Trypan Blue exclusion method and rubidium uptake. From other studies [for review see 6, 12] performed with the homologous 4-hydroxypentenal

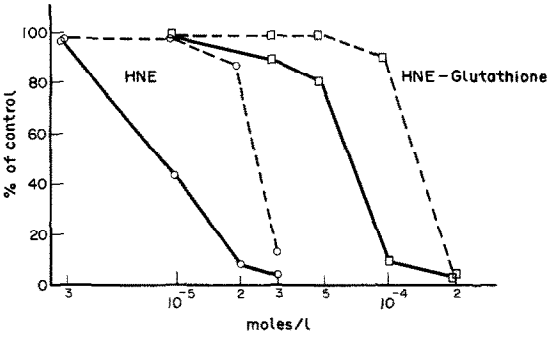

Fig. 4. Effect of 4-hydroxynonenal and 4-hydroxynonenal–glutathione on cell growth and viability. The cells were incubated for 45 hr in the presence of the drugs. 4-Hydroxynonenal: cell growth (O–O), viability (O· · ·O); 4-hydroxynonenal-glutathione adduct: growth (□–□), viability (□· · ·□); HNE = 4-hydroxynonenal.

it is known that such aldehyde concentrations do not influence glycolysis, respiration, protein synthesis or RNA synthesis. The inhibition of proliferation is most likely due to an inhibition of DNA synthesis. The evidence for this conclusion is based on the finding that (a) hydroxynonenal (20 μM) in fact blocks the *de novo* synthesis of DNA as shown by pulse label experiments and (b) inhibits the incorporation of externally added thymidine into the acid insoluble fraction without affecting the thymidine transport across the cell membrane. Further support for the conclusion that 4-hydroxynonenal directly interferes with DNA synthesis can be found in the literature: Seeber *et al.* [18] have shown using a cell free system that 4-hydroxypentenal inhibits the DNA polymerase as well as phosphorylation of nucleotides. Scaife [29] using the same aldehyde has found that it manifests its effects most readily on actively proliferating cells (kidney T cells, HeLa cells, mouse 3T3 cells) and less on nondividing cells (kidney T blocked in *S* phase, 3T3 contact inhibited). Dostal *et al.* [30] found that 4-hydroxypentenal (100 μM, 23 hr) inhibits incorporation of [^{3}H]-thymidine into chicken fibroblast cultures by 50%. The mechanism by which 4-hydroxynonenal and other 4-hydroxyalkenals exert their inhibitory action on DNA synthesis is not clear. The aldehydes have a high affinity to bind and thereby inactivate enzymes with essential SH groups. Since DNA polymerase possesses functional thiol groups [31] it is reasonable to assume that the blockage of the SH groups by the aldehyde plays an important role. This assumption is supported by the fact that low concentrations of 4-hydroxynonenal very selectively attack protein SH groups contained in the nucleus. After a 30 min incubation period of Ehrlich ascites tumor cells in the presence of 200 μM 4-hydroxynonenal the SH content of the soluble cytoplasmic proteins decreased only by 1.7% whereas the SH groups of the soluble nuclear SH proteins decreased by 27% [32]. Although no clearcut evidence exists, the finding described in this paper together with other reports strongly support the conclusion that 4-hydroxynonenal at low concentrations of about 10–20 μM selectively blocks the SH enzyme DNA polymerase. In addition, concentrations < 10 μM have a weak opposite effect, indicating an increased unscheduled DNA synthesis. The biological importance of these contrary influences on DNA synthesis by the biogenic 4-hydroxynenal is unclear.

An important factor for the use of anticancer drugs *in vivo* is their action on normal cells with high proliferating activity. A safe drug should inhibit growth of tumor cells without exerting cytotoxic effects on normal cells. A good experimental screening for the detection of cytotoxic and specific effects on cell proliferation is the cytostatic index (CI). The cytostatic index for 4-hydroxynonenal is 3.0, i.e. the concentration reducing cell viability by 50% is three times higher than the concentration leading to 50% inhibition of cell growth. Compared to the antitumoral N-Lost HN2, which has a CI of 100, 4-hydroxynonenal is therefore a less potent specific growth inhibitor. It has been shown [29, 30] that 4-hydroxyalkenals also inhibit growth of rapidly dividing normal cells; the observed inhibition of the DNA synthesis in Ehrlich ascites tumor cells caused by 4-hydroxynonenal would most likely also occur in normal cells with comparable doubling time. It was expected that the cytotoxic index could be improved by variation of the chemical structure of the aldehyde. The first approach was an elongation or shortening of the aliphatic side chain R of the aldehyde while maintaining the functional groups (R—CHOH—CH=CH—CHO). Among seven 4-hydroxyalkenals tested none had a CI better than 4-hydroxynonenal (CI = 3.0). In contrast all other CI were significantly lower (1.2–1.9). The superior position of the biogenic 4-hydroxynonenal among other 4-hydroxyalkenals, which are non biogenic, might in some way be connected with the capacity of the cell to detoxify the aldehydes by means of an enzymatic NADH dependent reductive process [24] and glutathione transferase [33]. A second approach was to use 4-hydroxynonenal in a masked form bound to cysteine or glutathione. These products were nearly one order of magnitude less toxic but since the growth inhibitory action was reduced to about the same extent the CI was not improved.

These results, however, show that in principal the possibility exists to design 4-hydroxynonenal derivatives which are less toxic than the free aldehyde but still possess growth inhibition activity. A great diversity of compounds possessing the functional active groups of 4-hydroxyalkenals (—CHOH—CH=CH—CHO) in free or masked form could be prepared by chemical synthesis and it seems reasonable to assume that among them are also compounds with improved cytostatic activity. Research in this field therefore, in the authors' opinion, opens new aspects for developing anticancer drugs.

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