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INVITED REVIEW

INTERACTION BETWEEN NEUTROPHILS AND 4-HYDROXYALKENALS AND CONSEQUENCES ON NEUTROPHIL MOTILITY

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The lipid peroxidation product 4-hydroxynonenal (HNE) and homologous aldehydes have been found to possess chemotactic activity for rat neutrophil leukocytes in the micromolar to picomolar range, depending on the compound. Such an activity is displayed only in the presence of albumin. The mechanisms by which aldehydes could interact with neutrophils are discussed. It is proposed that albumin acts as a carrier for the aldehyde and releases them to a neutrophil receptor. At concentrations around 10^{-4}M, 4-hydroxyalkenals have been found to exert toxic effects on a number of cells, including a strong depression of neutrophil motility. Finally, HNE has been found at chemotactic concentrations in the inflammatory site. The possibility that HNE is involved in the neutrophil influx into the inflammatory site is considered.

KEY WORDS: Aldehydes, chemotaxis, leukocytes, lipid peroxidation, cell motility, neutrophils, albumin.

INTRODUCTION

One of the earliest events in inflammation is the migration of neutrophil leukocytes from blood vessels into the injured tissue where they accumulate. Neutrophils are attracted to the site of injury by chemical substances diffusing from the site and stimulating a chemotactic response.† At higher concentrations, such as those reached at the site of injury, the same chemotactic substances inhibit the neutrophil movement. This phenomenon helps to trap neutrophils in the inflammed tissue.[2]

A wide variety of substances are reported to be chemotactic for neutrophils. These include proteins, peptides such as the complement derived C5a[3] and bacterial formylpeptides,[4] and lipids such as oxygenated products of polyenoic fatty acids. Turner *et al.*[5] were the first to demonstrate that the oxidation of fatty acids generates chemotactic stimuli. These Authors proposed that polyenoic fatty acids abundantly present in plasma membranes constitute a reserve of precursor of chemoattractants which would be produced when lipid peroxidation takes place. Successively, it was shown that the oxidation of archidonic acid through the lipoxygenase pathway in leukocytes leads to the formation of potent chemoattractants such as LTB_4 and various monohydroxyeicosatetraenoic acids.[6,7] In 1986, a novel lipoxygenase product was identified as 12-oxododeca-5,8,10-trienoic acid. Preliminary experiments in-

† The oriented migration of cells along a chemical gradient is called chemotaxis[1].

dicated that this C-12 unsaturated aldehyde possesses chemotactic activity on human neutrophils.[8]

Recently, evidence has been presented that 4-hydroxy-2,3-*trans*-alkenals constitute an additional class of chemoattractants.[9-11] The 4-hydroxyalkenals are biologically active aldehydes which are formed during the non-enzymatic peroxidative breakdown of polyenoic fatty acids.[12,13] From a biological point of view the most important 4-hydroxyalkenal is 4-hydroxynonenal (HNE), a high reactive compound which derives from the peroxidation of linoleic, γ-linolenic and arachidonic acid.[13] HNE was found at the picogram level in a variety of rat tissues,[14,15] probably produced during basal lipid peroxidation which might be a part of normal cellular metabolism. Moreover, HNE has been detected in larger amounts in peroxidized liver microsomes and isolated hepatocytes[14] as well as in the liver of bromobenzene-poisoned mice[16] and in degenerating retina[17] – two situations in which lipid peroxidation takes place. In addition, HNE has been found to be present in low density lipoproteins (LDL) of human serum exposed to oxidative conditions.[18] Lastly, HNE has been identified at micromolar concentrations in inflammatory exudates.[11,19,20]

4-Hydroxyalkenals were found to be able to stimulate the oriented migration of rat neutrophil leukocytes *in vitro* at very low concentrations (micro-picomolar, depending on the compound), whereas at higher concentrations they inhibit cell motility. The mechanisms by which these aldehydes could interact with neutrophils will be discussed, as well as their possible involvement in the recruitment of leukocytes in tissues affected by inflammatory or degenerative processes.

CHEMOTACTIC ACTIVITY OF 4-HYDROXYNONENAL AND HOMOLOGOUS ALDEHYDES. IMPORTANCE OF ALBUMIN

4-Hydroxynonenal and four homologous aldehydes with chain length between 8 and 15 carbon atoms (Figure 1) possess a moderate, dose-related chemotactic activity on rat neutrophils, as measured by a modified Boyden method and the leading front technique[9-11] (Figure 2).

The chemotactic activity of 4-hydroxyalkenals is expressed only in the presence of albumin. The influence of albumin is not attributable to impurities present in the used sample powder, since non purified bovine serum albumin (BSA) frac. V and pure BSA give the same results (Table I).

It is known that the potent chemoattractants formylpeptides, as well as other low molecular weight chemoattractants, require albumin (or serum) to express their chemotactic activity in a Boyden chamber or in an agarose plate.[21,22] It is not clear why the chemotactic activity of low molecular weight chemoattractants is dependent on albumin. It has been suggested that this protein acts as a carrier, presenting the chemoattractants to the cell membrane in a way which initiates a migratory response.[21] However, subsequent research has disproved this hypothesis.[23] Accurate studies have indicated that albumin supports cell movement by diminishing over strong adhesion of cells to the substratum.[24] However, Håkansson and Venge[25] recently conducted experiments which indicated that this is not the probable mechanism.

With regard to the 4-hydroxyalkenals, we can exclude the hypothesis that albumin supports their chemotactic activity solely by diminishing the cell adhesion to the substratum. We tested the ability of HNE to induce morphological polarization of neutrophils in suspension. This phenomenon is produced by chemotactic substances,

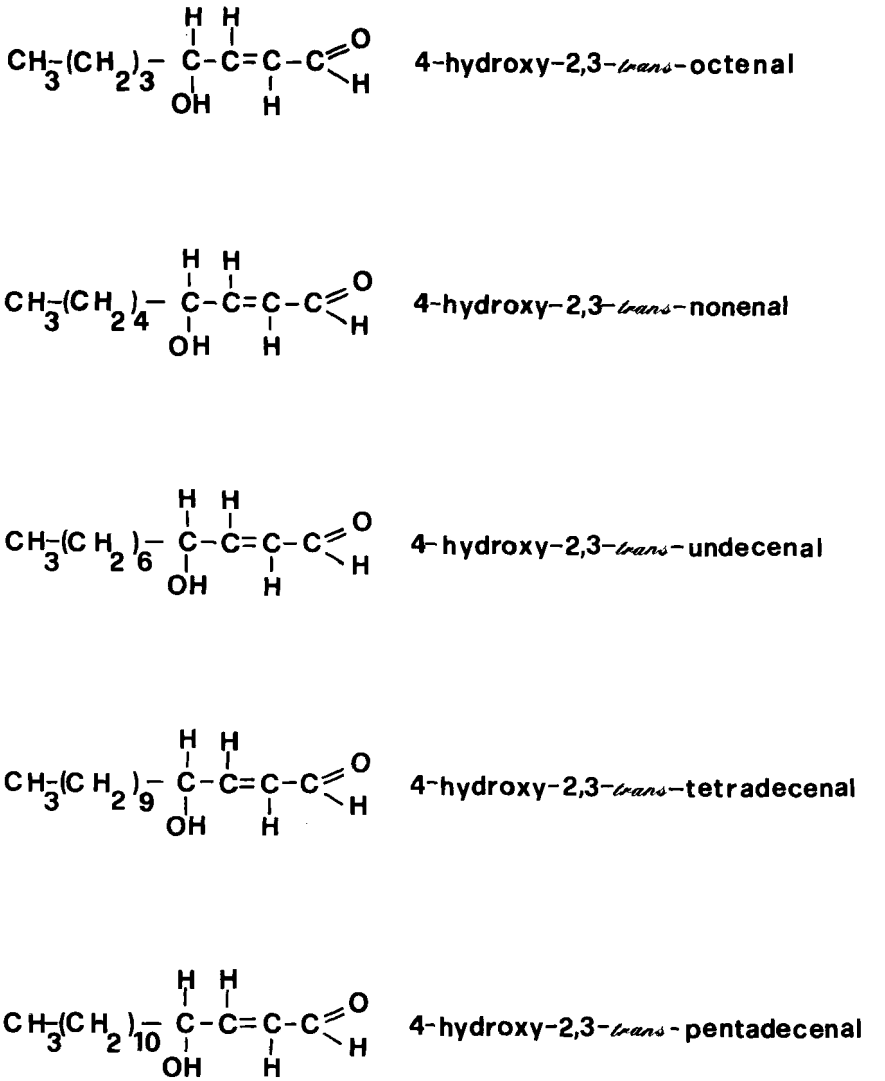

FIGURE 1 Structure of chemotactic 4-hydroxy-2,3-*trans*-alkenals.

is correlated with the locomotor function and does not involve adhesion to a substratum.[26] Results showed that, unlike other low molecular weight chemoattractants which induce polarization in the absence of albumin, HNE requires this protein to induce shape changes in neutrophils.[10] (and Curzio M., unpublished data).

α,β-unsaturated aldehydes are highly reactive electrophilic agents which in an environment near the neutral pH, easily react with sulfhydryl groups of various proteins.[12] By a 1,4-addition reaction an adduct is formed in which the SH-compound is bound at C-3 by a thioether linkage. In the case of 4-hydroxyalkenals the primary product cyclizes, forming a hemiacetal which is in equilibrium with the linear adduct (Figure 3). Reactions between 4-hydroxyalkenals and NH_2-groups have been rarely observed at neutral pH.[27]

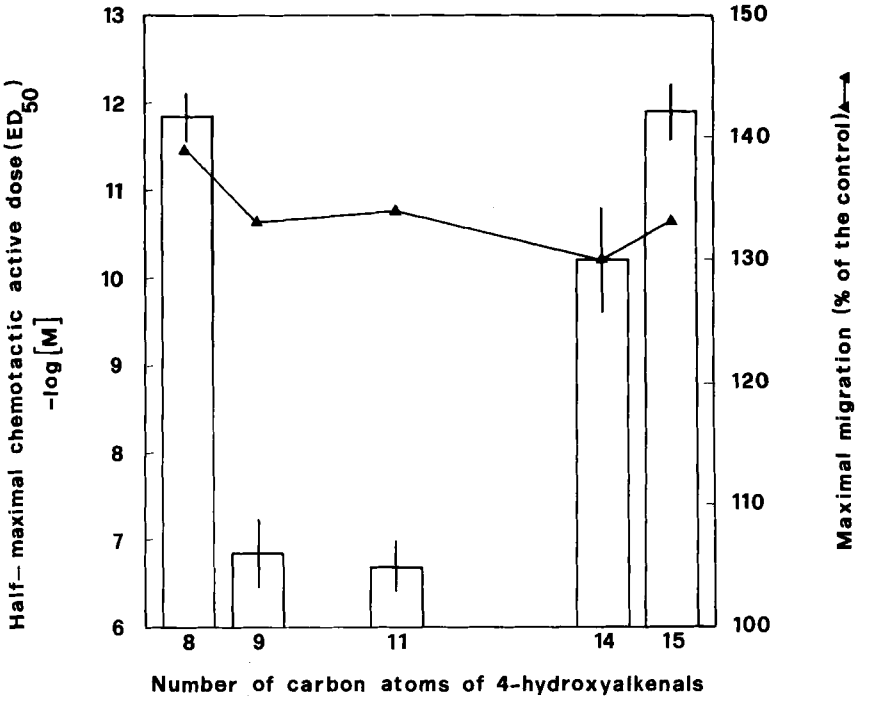

FIGURE 2 Chemotactic activity of 4-hydroxyalkenals towards rat neutrophils.Oriented migration of neutrophils stimulated by 4-hydroxyalkenals was measured by the Boyden methods as described.[10] The cells were diluted in Hanks' + 2% BSA and were placed in the upper compartment of the chambers, whereas the aldehydes, diluted in the same solution, were placed in the lower one. In the abscissa is given the carbon atom number of the 4-hydroxyakenals tested as chemoattractants. The histogram shows the concentrations of hydroxyalkenals required to produce 50% of the maximum effect determined by the concentration-effect curve. Each value is the mean ± standard error of at least three determinations with different neutrophil preparations. The line shows the maximal migration induced by the 4-hydroxyalkenals as compared with unstimulated control (100%). These data have been presented in more detail in Ref. 10

It is therefore possible that in our experimental conditions 4-hydroxyalkenals react with SH-groups of BSA and that the formed adduct is the chemotactic agent. In this connection it should be noted that albumin conjugated to various substances, including alkylating agents which attach non-polar group to the protein, is chemotactic for human neutrophils.[28] Albumin treated with a few aldehydes (formaldehyde, acetaldehyde, butyraldehyde) was found to be little or non chemotactic.[28] However, in these experiments albumin was conjugated to saturated aldehydes, which display a very different reactivity from 4-hydroxyalkenals. Saturated aldehydes first react with aminogroups of proteins, and then with other groups, inducing cross-linking and the formation of protein polymers.[12] Polymeric forms of proteins are deprived of chemotactic activity.[28]

The kinetics of the reaction between HNE and albumin in the physical-chemical conditions of the chemotaxis and polarization experiments was studied.[10] The reaction was found to be rapid and reversible, reaching an equilibrium after 60 min. In the presence of neutrophils the same reaction occurred, with only a small part of the HNE being bound to cellular components or metabolized. Since in the chemotaxis and polarization experiments the 4-hydroxyalkenals were diluted in the BSA-containing

TABLE I

Chemotactic activity of HNE towards rat neutrophils in the presence of pure bovine serum albumin (BSA) and fraction V BSA in the medium

Medium†	Stimulus	Distance moved by cells ($\mu \pm$ SD) in 75 min.	P	%
PBS‡ + pure BSA (A4378 Sigma)	None	62.00 ± 5.70	–	100
	fMLP (10^{-8} M)	87.00 ± 2.74	< 0.001	140
	HNE (10^{-6} M)	83.00 ± 4.83	< 0.001	134
PBS‡ + frac. V BSA (A4503 Sigma)	None	78.67 ± 9.35	–	100
	fMLP (10^{-8} M)	107.33 ± 8.21	< 0.001	136
	HNE (10^{-6} M)	101.67 ± 8.59	< 0.001	129

† Medium used to suspend cells and to dilute stimuli

‡ Phosphate buffered saline

Two experiments using two different cell preparations are shown. Cells and stimuli were diluted in PBS + 2% BSA, pH 7.2. Cells (5.5×10^6/ml) were added to the upper compartment of a Boyden chamber with stimulus in the lower one. Migration was measured by the leading front technique, with 5 counts for each filter.[9] Three filters for each stimulus were prepared. formyl-methionyl-leucyl-phenylalanine (fMLP) was tested as a comparison. The level of significance was measured by a *t* Student's test.

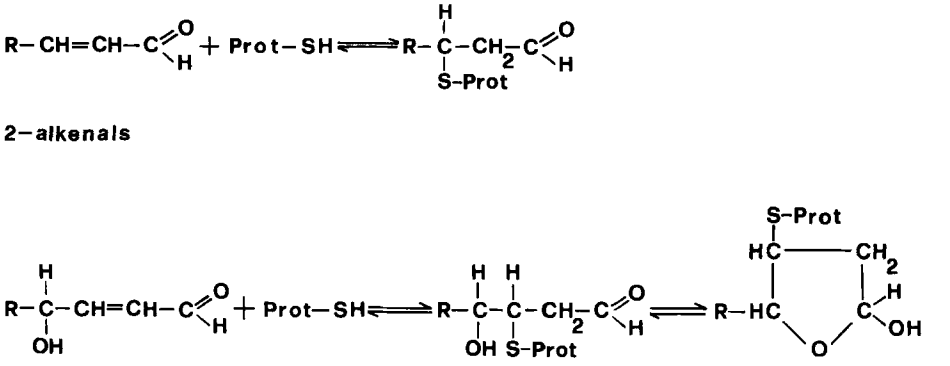

FIGURE 3 Reaction of α,β-unsaturated aldehydes with SH-groups in proteins.

buffer about 1 hour before being placed into contact with the cells, these cells were exposed to a solution containing mainly aldehyde bound to albumin.

Taken together, these observations suggest that the 4-hydroxyalkenals act as chemoattractants after being bound to albumin. However, this cannot be proved since the reaction between 4-hydroxyalkenals and BSA is reversible. Thus, a part of the hydroxyalkenal is always free in the medium and could itself interact with the cells.

The binding of albumin to aldehydes could condition their chemotactic activity by means of at least three different mechanisms:

1) The binding to the aldehyde could modify the tertiary structure of the protein and cause the exposure of previous intrinsic sites which would be recognized by cells. This hypothesis does not seem to be acceptable, since it does not explain why 4-hydroxyalkenals possess different chemotactic potency, even though they show similar chemical

reactivity and should alkylate albumin at the same site and to the same extent.[10] As can be seen in Figure 2, the chemotactic effective concentrations of various 4-hydroxyalkenals differ by five orders of magnitude.

2) Albumin conjugated to 4-hydroxyalkenals would be recognized by a receptor present on the cell membrane and containing multiple hydrophobic sites. Hydrophobic reactions could be established with non-polar aliphatic chains of hydroxyalkenals bound to albumin. Intrinsic groups of the protein exposed as a consequence of the modification of its tertiary structure might strengthen the bond. A similar mechanism has been proposed as being involved in the recognition, by neutrophils, of the chemotactic albumin conjugated to various non-polar compounds.[28] However, the chemotactic potency of the 4-hydroxyalkenals tested is not directly related to their hydrophobicity, the most active compounds being the most hydrophobic and the least hydrophobic ones[10] (Figure 2). Therefore, the hypothesis of a hydrophobic receptor for the adduct does not explain the data either. However, we cannot exclude the possibility that this mechanism is involved in the chemotactic activity of the most hydrophobic aldehydes, whereas the least hydrophobic ones would act through another mechanism.

3) Albumin could act as a carrier of the aldehydes and exhibit them in the right position for an easy interaction with an SH-cellular molecule. Such a molecule should have a higher affinity for the aldehydes than albumin. Less probably, 4-hydroxyalkenals could be exhibited to an NH_2-compound. Such a high affinity receptor might also contain a non-polar part to establish hydrofobic interactions with the aliphatic chain of the aldehydes and then stabilize the bond. Moreover, to explain the finding that the most powerful aldehydes are the most hydrophobic and the least hydrophobic ones, whereas aldehydes of intermediate polarity are less potent, two receptors, one containing a larger, the other a smaller non polar-part, could be postulated.

INHIBITORY EFFECTS OF HNE AND HOMOLOGOUS ALDEHYDES

4-Hydroxyalkenals, put in the lower or in both the compartments of a Boyden chamber at concentrations around 10^{-4}M, strongly depress random and oriented migration of rat[10] and human neturophils[29] (Figure 4). This effect was observed both in the presence and in the absence of albumin in the medium. The inhibition of neutrophil migration shown by 4-hydroxyalkenals cannot be ascribed to an impairment of cell viability,[10,29] but it is probably related to a depression of some metabolic functions involved in the locomotion. To clarify this point, it seems necessary to investigate what happens when neutrophils are exposed to high concentrations of aldehydes. 4-Hydroxyalkenals could react with SH-molecules (or less probably with NH_2-molecules) contained in the plasma membranes and involved in the locomotion, whether or not they are specific receptors for aldehydes. Alternatively, 4-hydroxyalkenals could enter into the cells by diffusion and reach a concentration high enough to block sulfhydryl groups of molecules essential for neutrophil motility,[30] for example the microtubular protein tubulin. It is known in fact that 4-hydroxyalkenals are able to damage tubulin *in vitro*.[31]

Interactions with SH-groups of cellular molecules have also been proposed as causing the inhibitory effect on neutrophil motility exerted by other unsaturated aldehydes (acrolein, crotonaldehyde) which are present in tobacco smoke. Like HNE, these aldehydes were found to be inhibitory in the 10^{-4}M range.[32] Evidence has been

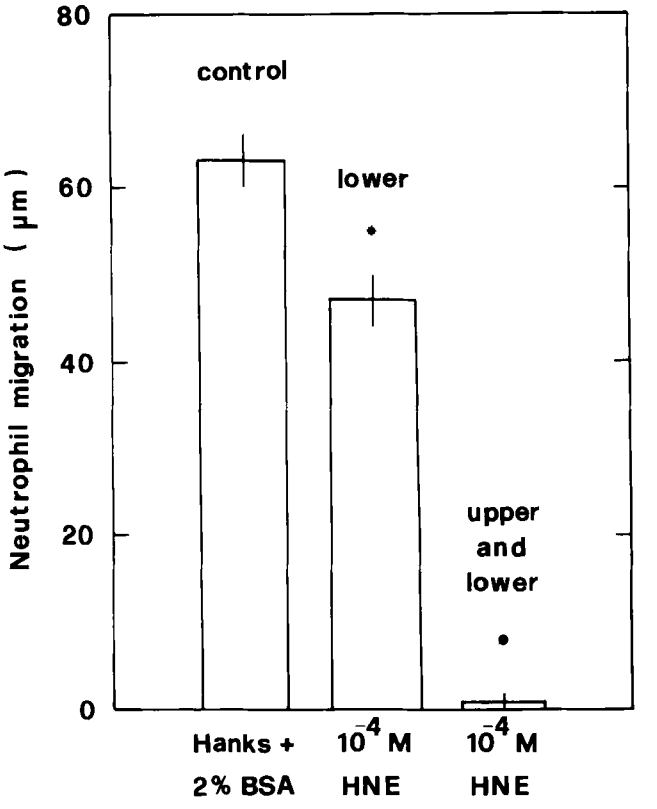

FIGURE 4 Inhibition of rat neutrophil migration by 10^{-4}M HNE. Neutrophils were collected from the rat pleural cavity as described,[10] then washed and placed in the upper compartment of a Boyden chamber. Cell migration in the presence of 10^{-4}M HNE in the lower compartment or in both the compartments of a Boyden chamber was measured as described.[10] HNE was diluted in Hanks' + 2% BSA. Migration is expressed as microns moved into filters by the cell front during 75 min. of incubation. As a control, neutrophil random migration in the absence of HNE was measured. Level of significance versus control was determined by a *t* Student's test. * = $P < 0.001$.

presented showing that their effect is related to interactions with SH-compounds, but they have not been identified.

Moreover, it is interesting to note that at concentrations similar to those we found to inhibit neutriohil migration, 4-hydroxyalkenals exert a number of toxic effects not only on neutrophils but also on a variety of cells. With regard to neutrophils, 10^{-4}M HNE completely inhibits stimulated O_2^- production.[33] With regard to other inflammatory cells, 10^{-4}M 4-hydroxyoctenal and 4-hydroxynonenal induce a strong inhibition of monocyte immune phagocytosis.[34] In addition, it has been observed that HNE inhibits protein, RNA and DNA synthesis, and the expression of the c-myc oncogene in K562 cells[35] as well as the activity of DNA-polymerase from rat liver.[36] Lastly, five 4-hydroxyalkenals induce DNA fragmentation and sister-chromatid exchanges in ovary cells.[37] A review of the many toxic effects of aldehydes is given in several reports.[12,38,39] The variety of toxic effects observed suggests that manifold mechanisms underlie the activity of 4-hydroxyalkenals at high concentrations.

POSSIBLE INVOLVEMENT OF HNE IN INFLAMMATORY EVENTS

A series of observations suggest that HNE could be involved in the neutrophil recruitment at the inflammatory site. First, HNE has been identified in rat exudates formed in the pleural cavity after the intrapleural injection of isologous serum[11,19] and in subcutis after the subcutaneous injection of Sephadex.[20] Second, the HNE peak concentration in the exudates was around micromolar in both these experimental models. At this concentration the aldehyde is chemotactic *in vitro* for rat neutrophils. Third, the albumin concentration in any exudate is certainly high enough to support the chemotactic activity of HNE. Fourth, by using the pleurisy model, it was observed that neutrophils and HNE increased in parallel in the exudate during the experimental period.[11] When the Sephadex model was used accumulation of HNE preceded the neutrophil peak.[20] However, the hyphotesis that HNE acts as a chemoattractant *in vivo* will require further critical testing.

The presence of HNE in the exudate is probably a consequence of lipid peroxidation reactions which take place in the inflammatory site.[40] The finding that various lipid peroxidation products, such as n-alkanals, increase in parallel to HNE in the exudate during the experimental pleurisy[11] supports this hyphotesis. The lipid peroxidation reactions could be triggered in the inflammatory area by oxygen radical metabolites produced by phagocytes and released in part into the surrounding tissue. Thus, HNE could be generated by lipid peroxidation reactions occurring in damaged neutrophils as well as in other tissue cells. Experiments performed by Kink *et al.* indicate that stimulated production of O_2^- by neutrophils is correlated to HNE production by the same cells.[20] However, neutrophils collected from the rat pleural cavity 4 hours after a pleurisy induction and incubated at 37°C for 60 min. in a hyperoxygenated environment were found to be unable to produce HNE (Curxio M. and Biasi F., preliminary results).

It remains to explain why malondialdehyde, a compound formed in large amounts during lipid peroxidation reactions, was not found to increase during the experimentally induced pleurisy.[11] A different catabolism of the different aldehydes could be suggested to explain this observation.

Another important point is that the adduct of HNE with albumin is in equilibrium with the free aldehyde.[10] So this may be a mechanism for transporting the aldehydes produced in the inflammatory site to distant areas. We are aware that several general reactions occur in an organism as a consequence of the presence of an inflammatory focus. Some of these reactions occur in the liver where the so-called acute phase proteins are produced. It has been shown that the preferential synthesis of these proteins is the consequence of the activation of their coding genes. As HNE is able to act on DNA functions,[35,37] another point to investigate is if the production of the acute phase proteins may depend upon the aldehydes released at the inflammatory site.

POSSIBLE INVOLVEMENT OF HNE IN SOME DEGENERATIVE PROCESSES

Evidence has recently accumulated suggesting that many of the foam cells found in atherosclerotic lesions are derived from monocytes/macrophages.[41] The exact mechanism by which monocytes are recruited in the arterial wall is unknown, but probably

chemotactic factors are involved in the phenomenon. In 1987 Quinn *et al.* demonstrated that oxidatively modified LDL are chemotactic for human monocytes.[42] They further showed that the chemotactic activity resides predominantly in one or more of the peroxidized lipid components of LDL. In addition, it has been found that macrophages take up and degrade oxidatively modified LDL at much higher rates than native LDL.[43] Therefore, if operative *in vivo*, oxidative modification of LDL may contribute to atherogenesis both by influencing the recruitment of monocytes in the subendothelial space and by favoring the accumulation of cholesterol stores in developing foam cells. The relevance in atherogenesis of oxidative modification of LDL is supported by studies showing that probucol, which prevents oxidation of LDL *in vitro*, prevents the progression of atherosclerosis *in vivo* in Watanabe heritable hyperlipidemic rabbits.[44] Since HNE has been unequivocally identified in LDL particles exposed to oxidative conditions,[18] the possibility exists that this aldehyde confers the observed chemotactic activity for leukocytes to the particles. Moreover, the incubation of LDL with HNE enhances LDL uptake by macrophages.[45] Thus, HNE could be involved in the complex process of atheroscolerosis.

In addition, HNE might be involved in the development of another degenerative disease, retinal phototoxical degeneration. As mentioned above, HNE has been identified in retina of rats exposed to phototoxic stress.[17] The same retina showed extensive loss of photoreceptors cells, whereas migratory cells, probably phagocytes, were present in the subretinal space. Thus, it can be suggested that the migratory cells have been attracted by HNE produced by lipid peroxidation reactions in the retina.

FUTURE ASPECTS

Further research is needed to establish whether the chemotactic activity of 4-hydroxyalkenals on neutrophils is mediated by specific receptors which recognize the aldehyde, or the aldehyde-albumin adduct, or by an unspecific mechanism. Recently, sinusoidal liver cells, probably Kupffer cells, were found to possess specific receptors on the plasma membranes which recognize the adduct formed by saturated aliphatic aldehydes and several proteins, including albumin. The reaction of lysyl residues of proteins with aldehydes would be involved in the formation of the active ligand.[46] However, as stated above, the most acceptable hypothesis to explain the first interaction between 4-hydroxyalkenals and neutrophils is that it is mediated by a receptor for the aldehyde, rather than by a receptor for the albumin-aldehyde adduct.

The low concentrations at which aldehydes act as chemoattractants suggest the presence of a high-affinity receptor on neutrophils. Another clue for the presence of a saturable receptor for 4-hydroxyalkenals is that homologous, but not heterologous, deactivation can be induced by preincubating cells with high concentrations of aldehydes.[47] Down regulation of specific receptors could explain this finding.

Such a binding site would not necessarily recognize only 4-hydroxyalkenals. Studies were undertaken to determine the relationship between the structure and activity of different classes of aldehydes. Alkanals and 2-alkenals of the same chain length of chemotactic 4-hydroxyalkenals were tested for their ability to induce a chemotactic response in rat neutrophils in the same experimental conditions as 4-hydroxyalkenals.[11] Saturated n-alkanals, differing from 4-hydroxyalkenals lacking the *trans* C–C double bond and the OH group, were observed to be deprived of chemotactic activity. 2-Alkenals, differing from 4-hydroxyalkenals only in their lack of the OH

group, were found to possess a dose-related chemotactic activity. The extent of stimulation induced by each 2-alkenal is similar to that induced by the analogous 4-hydroxyalkenal. These results indicate that the OH group is not a necessary requirement for the recognition of aliphatic aldehydes by neutrophils, and nor is the formation of cyclic adduct with albumin. It also appears clear that the unsaturated C–C double bond conditions the chemotactic activity of the aldehydes.

Moreover, the 2-alkenals tested (2-octenal, 2-nonenal) were found to act at lower concentrations than 4-hydroxyalkenals of the same chain length. This might be related to the higher instability of the adduct formed by 2-alkenals with albumin rathen than by 4-hydroxyalkenals.[12] In fact, it would be easier for albumin to release 2-alkenals to the supposed receptor rather than 4-hydroxyalkenals.

Experiments have been planned in our laboratory to investigate whether or not receptors for α,β-unsaturated aliphatic aldehydes are present on neutrophil leukocytes.

We should also like to see whether the postulated receptors are expressed on all cells, whether antagonists could eventually occupy them and whether other substances could modulate their expression. The raison for our interest is that the stimulation of oriented migration induced by 4-hydroxyalkenals and 2-alkenals is weaker than with other known chemoattractants[10,11] and because we observed that some preparations of rat neturophils do not respond chemotactically to the aldehydes.[10,11]

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