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THE LIPID PEROXIDATION PRODUCT 4-HYDROXYNONENAL IS FORMED BY - AND IS ABLE TO ATTRACT - RAT NEUTROPHILS *IN VIVO*

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4-Hydroxynonenal (HNE), a major aldehydic product of lipid peroxidation, is a chemoattractant for neutrophilic polymorphonuclear granulocytes *in vitro*. The question was studied, whether HNE is formed during the ingress of neutrophils in the Sephadex model of inflammation. The polydextrane Sephadex G-200, which causes an acute aseptic traumatic inflammation, was injected subcutaneously into rats. The implants were excised 6-36 hours later, and the neutrophils separated from the exsudate by centrifugation. After extraction with dichloromethane HNE was identified in the exsudate by non-derivative reversed phase HPLC in combination with on-line uv-spectroscopy. The concentration of HNE in the inflammatory focus did not correlate with the number of neutrophils present. While the peak of HNE coincided with the time point of the highest turnover rate of neutrophils (0.13 μM at 6 hrs after implantation), the highest number of neutrophils (about 100 million cells) occurred not earlier than 18 hrs later (24 hrs after onset of inflammation).

When neutrophils were isolated from the inflammatory focus and stimulated with Zymosan, they were able to produce HNE *in vitro* depending on the time of isolation. The highest production of HNE (0.17 μM) by phagocyting neutrophils was observed at the shortest inflammation time studied (3 hrs). In order to compare these results with the oxidative burst of neutrophils the formation of superoxide was also measured by the cytochrome c reduction assay *in vitro*. The maximum of the production rate of superoxide anion was observed at the same inflammation time (6 hrs), when the HNE maximum occurred. Cells which ingressed earliest (at 3 hrs) showed the highest production rate of superoxide per cell (307×10^{-18} moles per cell and 30 min).

The ability of HNE to attract neutrophils *in vivo* was studied by adding synthetic HNE to the Sephadex gel and measuring the ingression of neutrophils afterwards. The application of 1 μM HNE in the focus did not change the number of neutrophils but 10 μM HNE increased the cell number by a factor of 3.

The results indicate that HNE is not only a chemoattractant for rat neutrophils *in vitro* but also *in vivo*. It is suggested that HNE is produced by selfdestruction of neutrophils during a traumatic inflammation and its production seems to be tightly connected to the oxidative burst of neutrophils. The idea of HNE as part of an autocatalytic cycle is supported whereby neutrophils which immigrate into an inflammatory focus produce HNE which stimulates the ingress of new neutrophils.

KEY WORDS: 4-hydroxynonenal, sephadex inflammation model, chemotactic activity, neutrophil granulocytes.

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INTRODUCTION

A wide variety of substances are reported to be chemotactic for neutrophil polymorphonuclear granulocytes (PMN) including proteins, peptides such as the complement derived C5a and bacterial formylpeptides, and lipids such as oxygenated products of polyenoic fatty acids.[1] Turner *et al.*[2] 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.

The lipid peroxidation product 4-hydroxynonenal (HNE) which is derived from omega-6-polyunsaturated fatty acids, such as linoleic, linolenic and arachidonic acid, shows several remarkable biological properties (for reviews see Zollner *et al.*[3]; Schaur *et al.*[4]) including the ability to stimulate the oriented migration of rat neutrophils *in vitro* at micromolar concentration or less.[1] It was also detected in pleural exsudates of rats[5] indicating its occurence during inflammation *in vivo*. In this paper the question was studied, whether HNE is formed during the ingress of neutrophils in the Sephadex model of inflammation. Furthermore the ability of HNE to attract neutrophils *in vivo* was investigated. *In vitro* experiments were designed to identify the cells of origin of HNE and to explore the relationship between the ability of neutrophils to produce HNE and the oxygen burst (formation of superoxide anion).

MATERIAL AND METHODS

Elicitation of Inflammation and Isolation of Neutrophils

The Sephadex model was used to produce an acute aseptic traumatic inflammation.[6] Briefly, 5 ml of swollen (3 days at room temperature) and sterilized (45 min/110°C) Sephadex G-200 (Pharmacia, Sweden) suspension (4% w/v in saline) were injected subcutaneously into the subcutis of the right lower back quadrant of ether-anesthetized Sprague Dawley rats with a weight range of 250–300 g.

After various time intervals the rats were anesthetized with ether and decapitated. The Sephadex gel containing the exsudate with neutrophils was collected with 10 ml saline into a petri dish, the gel was removed by filtration through a filter (Schwarzband, Schleicher & Schüll, Germany) and the retentate was washed with 10 ml saline. The filtrate and the washing solution were combined and termed "filtrate 1".

After centrifugation of filtrate 1 (5 min, 150 × g) the supernatant ("supernatant 1") was used for the determination of HNE; the pellet ("neutrophil pellet 1") was used for cell counting.

Counting of Neutrophils

The neutrophil pellet 1 was resuspended in saline for cell counting in a Coulter-Counter. For the determination of the percentage of neutrophils a differential staining according to Mey-Grünwald was carried out. The viability was checked with the trypan exclusion test. The percentage of unstained cells was always higher than 90%.

Separation, Identification and Quantification of HNE

HNE was extracted from the supernatant 1 by 40 ml dichloromethane on an Extrelut™ (11 738 Merck, Germany) solid phase column after 10 min of equilibration.

From the organic phase HNE was transfered into 2 ml of an acetate buffer (pH 3,5–4) by rotatory evaporation. HNE was identified by non-derivative reversed phase HPLC according to Lang *et al.*[7]

Separation conditions: Chromatograph Du Pont 838 with a pre-separation column Microprep RP18 30 μ and a separating column Spherisorb ODS 5 μ; injection volume: 1 ml; elution by 55% (v/v) acetonitrile in water; uv detection wave length: 223 nm; molar extinction coefficient epsilon: 13 750 l/mol × cm; the detection limit was about 0.03 μM.

For the recording of the spectra a Hewlett-Packard diode array detector model 1040A was used.

Application of Synthetic HNE

Synthetic HNE was added in a concentration of 1 and 10 μM resp. to the Sephadex gel before injection. In the case of 1 μM HNE 3 animals were used for the experimental group and for the control group, resp. In the case of 10 μM HNE the number of animals in each group was 5.

The elicitation of inflammation and the isolation of neutrophils were carried out as described above.

Determination of HNE in Stimulated Neutrophils in vitro

The potential of neutrophils to produce HNE during phagocytosis *in vitro* was studied by incubation of cells in presence of opsonized Zymosan[8] and measuring the HNE produced in dependance on the inflammation time.

Opsonization was carried out with serum of the same animal. After anesthesia 2 ml blood were obtained from the carotis and centrifuged (5 min, 500 × g). 1 ml serum was incubated for 15 min with 1 mg Zymosan at 37°C.

The opsonized Zymosan suspension was incubated for 35 min at 37°C with the neutrophil pellet 1 which was obtained at various time intervals and resuspended in 20 ml Hanks' solution. Immediately after incubation this cell suspension was directly applied on top of an Extrelut column for the determination of HNE.

Determination of the Superoxide Production by Neutrophils in vitro

The ability of neutrophils to produce activated oxygen species in the course of inflammation was studied by the determination of superoxide anion formation by the method of Babior *et al.*[9] The reduction of cytochrome-c within 30 minutes was used to measure the amount of superoxide produced in cells which were collected after various time intervals of inflammation.

4 ml samples of filtrate 1 were mixed with 2 ml Hanks' solution and 40 μl cytochrome c (10 mg/ml, type III, Sigma, USA). For the determination of the zero time point value an aliquot of 3 ml was immediately placed on ice. The remaining 3 ml were incubated for 30 min at 37°C and then also placed on ice. All samples were centrifuged at 4°C and the spectrum recorded in the range from 525 to 575 nm.

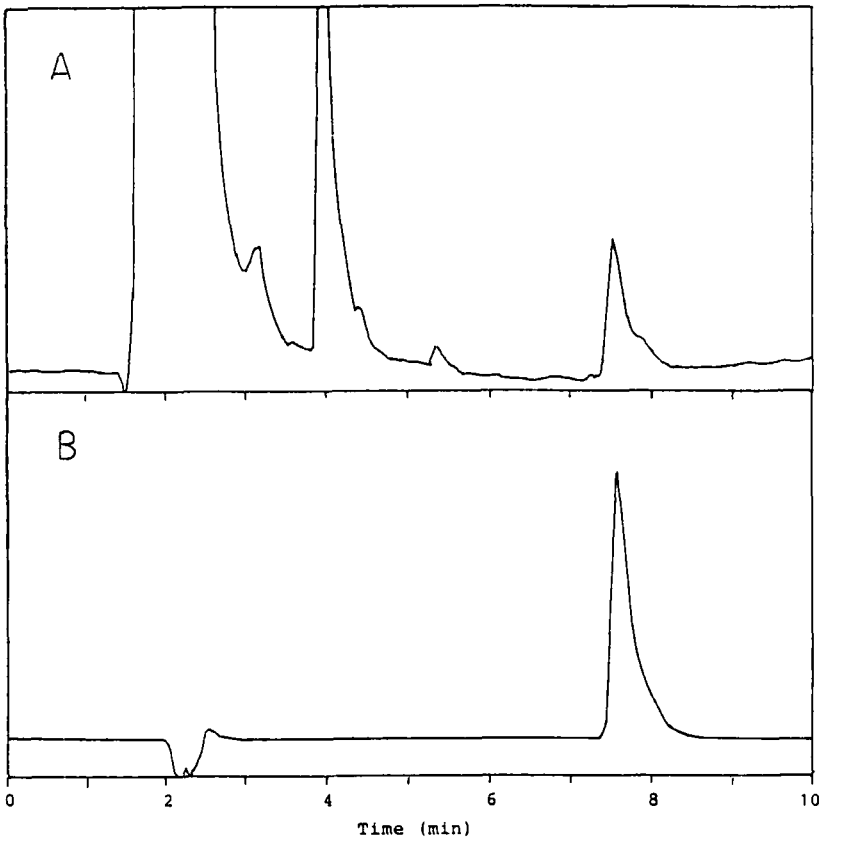

FIGURE 1 HPLC-chromatogram of HNE. A: chromatogram of an extract from the inflammatory site at the 6th hour after onset of inflammation. Attn (mAU): 10. B: chromatogram of synthetic HNE (0, 1 mM). Attn (mAU): 200. Separation conditions as described in the Material Section.

The peak extinction at 550 nm was evaluated. In order to correct for unspecific cytochrome c reduction parallel samples were run to which 0, 1 ml superoxide dismutase (3 mg/ml, Sigma, USA) were added.

RESULTS

Identification of HNE

HNE was identified by the HPLC chromatogram (Figure 1) and the corresponding on-line spectrum (Figure 2). Chemically synthesized HNE was used as a reference. Both compounds had identical retention times on the reversed phase HPLC column and showed an ultra violet absorption maximum at the identical wave length of 225 nm.

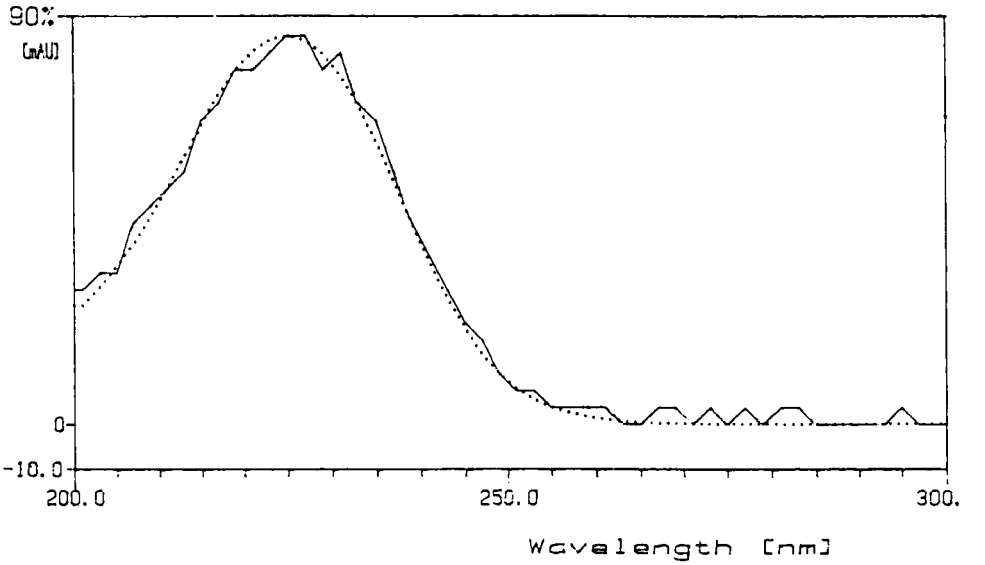

FIGURE 2 On-line spectrum of HNE. The spectra were recorded on line from the HNE peaks shown in figure 1. Full line: biogenic HNE; absorption maximum 225 nm; Attn (mAU): 3.3. Dotted line: synthetic HNE; absorption maximum 225 nm; Attn (mAU): 154.

Dependence of HNE Formation in vivo *on Inflammation Time*

Figure 3 shows the time dependance of the occurrence of neutrophils and HNE in the inflammatory focus. The maximum of the HNE concentration (0.134 μM, corresponding to 138×10^{-18} moles per single cell) occurred distinctly earlier (6 hrs after gel implantation) than the maximum of the number of neutrophils at the site of inflammation (24 hrs). Cells which immigrated earliest, produced the highest amount of HNE (810×10^{-18} moles per cell at 3 hrs after onset of inflammation).

Effect of Synthetic HNE

Synthetic HNE was added in a concentration of 1 and 10 μM resp. to the Sephadex gel prior to injection. While 1 μM HNE did not change the number of neutrophils found after an inflammation time of 6 hrs in the gel, the number of neutrophils was 3-fold higher than in the untreated control animals, when 10 μM HNE was applied ($23.2 +/- 10.5 \times 10^6$ cells versus $7.5 +/- 1.8 \times 10^6$ cells; $n = 5$). A double-sided Student's t-test showed a highly significant difference of the mean values ($2P = 0.01$).

Formation of HNE by Stimulated Neutrophils in vitro

The dependance on inflammation time of HNE formation by phagocytizing neutrophils *in vitro* is shown in Figure 4. The highest production of HNE (0.17 μM) by phagocytizing neutrophils was observed with cell obtained 3 hours after the onset of inflammation. This is a twofold increase when compared with the value obtained

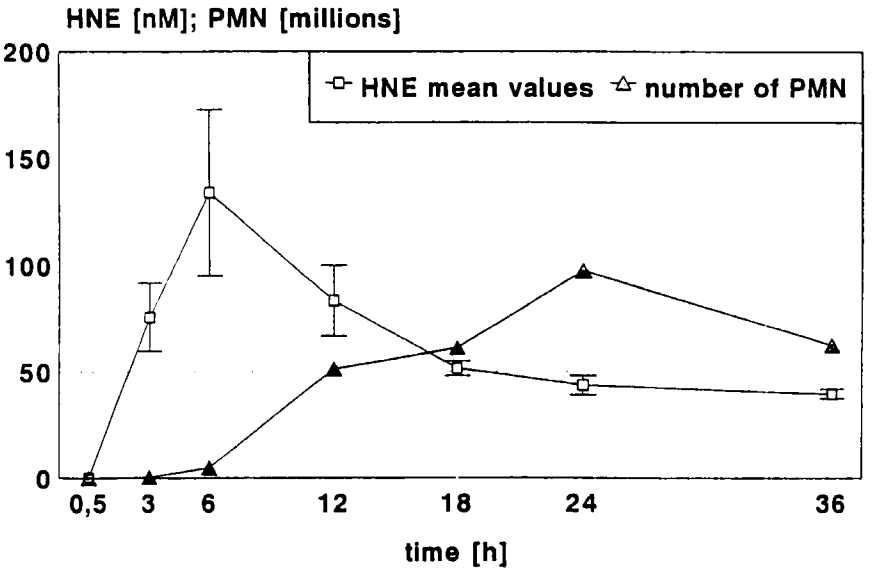

FIGURE 3 Time course of neutrophils and HNE in the inflammatory focus. At zero time an inflammation was provoked in rats by injection of Sephadex gel and the concentration of HNE and the number of neutrophils in the inflammatory focus were determined; time intervals (hrs) and number of animals (in brackets): 0.5 (1); 3 (3); 6 (4); 12 (3); 18 (3); 24 (3); 36 (3).

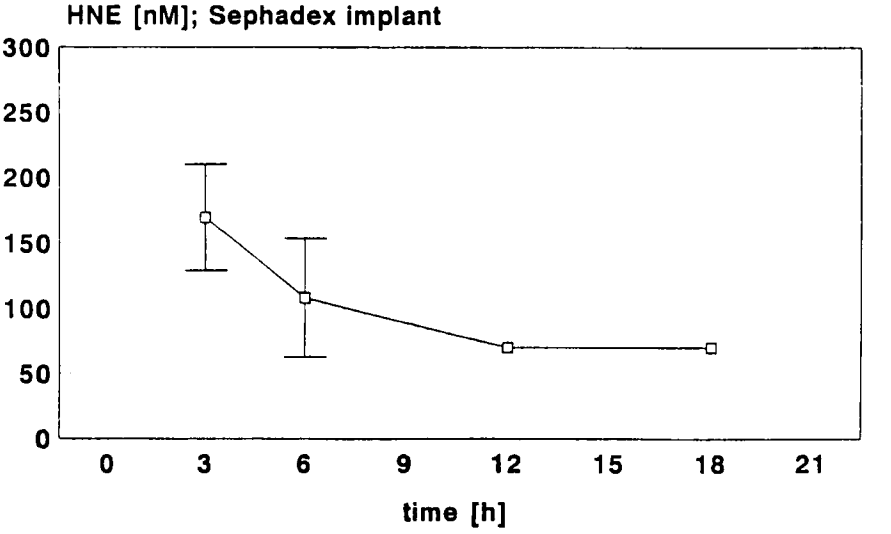

FIGURE 4 Formation of HNE by stimulated neutrophils *in vitro*: dependance on inflammation time. At zero time an inflammation was provoked in rats by injection of Sephadex gel and at the time intervals indicated the neutrophils were collected, stimulated with Zymosan to phagocytosis and incubated at 37°C. 35 min after stimulation HNE was determined. For the reason of comparison the concentrations given refer to the Sephadex implant; time intervals (hrs) and number of animals (in brackets); 3 (3); 6 (3); 12 (2); 18 (1).

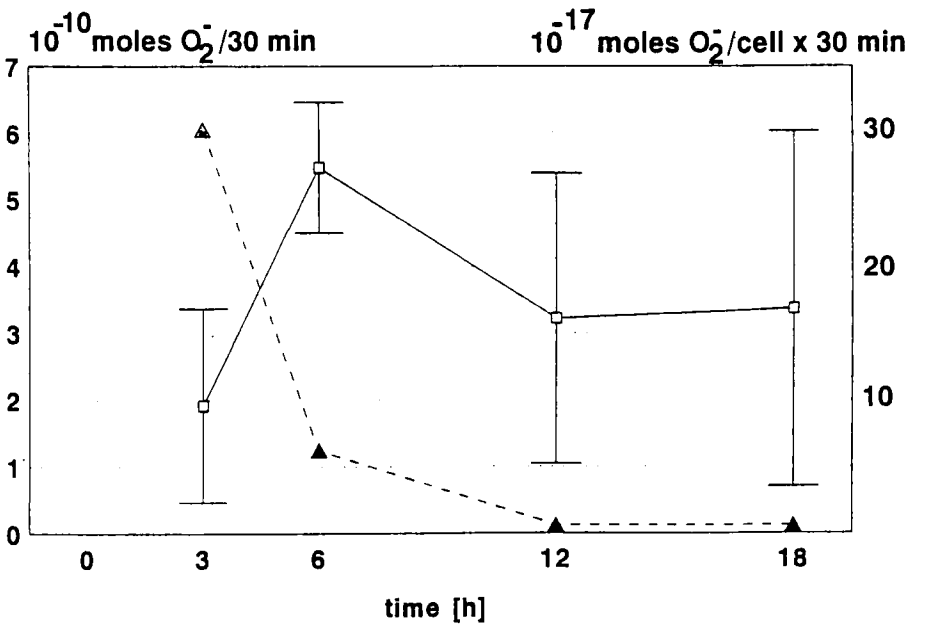

FIGURE 5 Production rate of superoxide by neutrophils *in vitro*: dependance on inflammation time. At zero time an inflammation was provoked in rats by injection of Sephadex gel and at the time intervals indicated the neutrophils were collected; the production rate of superoxide anion was determined for an incubation period of 30 min at 37°C; full line: total production of superoxide anion within 30 min; broken line: production within 30 min per single neutrophil.

with non-stimulated cells after 3 hours of inflammation *in vivo* (0.076 μM; see Figure 3).

Superoxide Production by Neutrophils in vitro

Figure 5 shows the dependance on inflammation time of the superoxide anion production by neutrophils *in vitro*. The maximum of the production rate of superoxide was observed with cells obtained at the same inflammation time (6 hrs), as was the case for the maximum of the HNE concentration (see Figure 3). The increase of the production rate between the value at 3 hrs (1.92×10^{-10} moles/30 min) and 6 hrs (5.48×10^{-10}/30 min) resp. was found to be significant, as indicated by a two-tailed Mann-Whitney test ($2P < 0.05$). Cells, which immigrated earliest, showed the highest production rate of superoxide per cell (307×10^{-18} moles per single cell and 30 min).

DISCUSSION

It has been established by Shohet *et al.*[10] and Stossel *et al.*,[11] that lipid peroxidation does occur in neutrophils. We have shown recently that the lipid peroxidation product HNE is produced in the micromolar range from liposomes by a pathway which depends on myeloperoxidase isolated from neutrophils.[12]

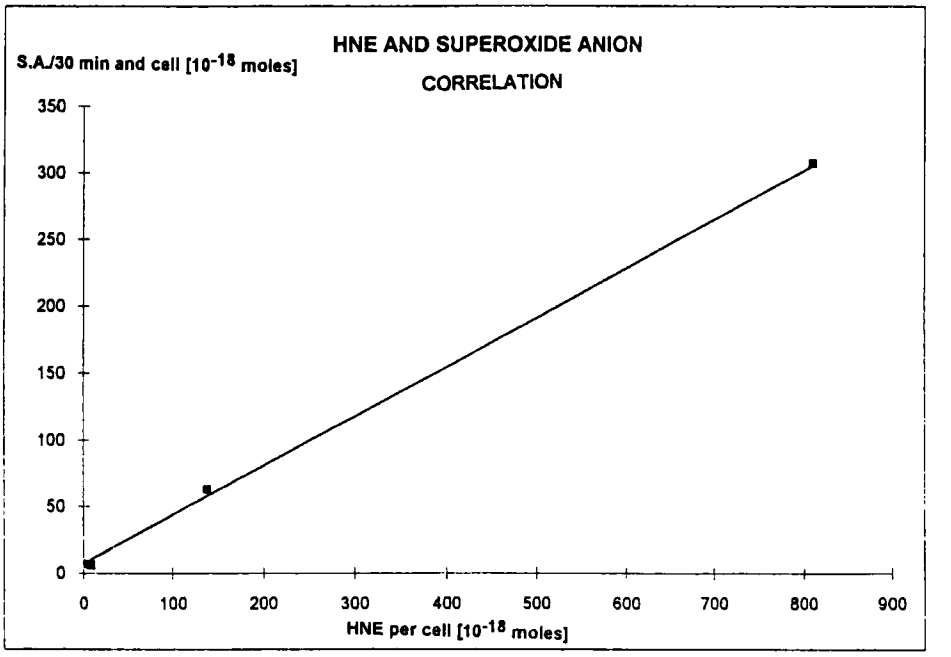

FIGURE 6 Correlation between HNE and the production rate of superoxide. A correlation study was performed using the data of figures 4 and 5; a highly significant correlation was found between the superoxide anion production rate (ordinate) and the amount of HNE formed by these cells (abcissa); $r = 0.999$; $2P < 0.002$).

In this study HNE was identified at the site of an acute traumatic aseptic inflammation. Its concentration was found to be sufficiently high to show a chemotactic effect *in vitro*.[1] The HNE concentration as determined by the method used reflects unreacted HNE only, while another part of the total HNE formed is simultaneously metabolized. The unreacted fraction can be regarded as essential for chemotaxis, since it has been shown by Curzio,[1] that the conjugated carbonyl structure, which is lost during reactions, is needed for this activity.

In search for the origin of HNE it was observed, that stimulated neutrophils were able to produce more HNE *in vitro* than was found in the exsudate. It is concluded that neutrophils contribute to the process of HNE formation during inflammation. An explanation for the relatively early maximum of the HNE concentration at the site of inflammation (6 hrs) compared with the peak of neutrophils (24 hrs) can be obtained by taking into account the time dependance of the turn over rate of neutrophils. At about 6 hrs after onset of inflammation both the rates for ingress (52 million cells per hour) and decay of neutrophils show a peak resulting in a minimum of half life (about 20 min).[13] This suggests that HNE is produced by the process of autoxidative selfdestruction of neutrophils.

When applied exogenously HNE was able to augment the number of neutrophils in the inflammatory focus. The concentration necessary for this *in vivo* effect was considerable higher than the effective concentration for chemotaxis *in vitro*. The concentration for half maximal stimulation of oriented migration *in vitro* was found to be 0.14 μM.[14] The difference may be due to the fact that most likely the greater

part of HNE is retained within the Sephadex particles, while the neutrophils accumulate in the outer shell of the gel. Moreover at the boundary of the gel to the surrounding tissue HNE will diffuse away so that more HNE is need to build up a gradient comparable to stable *in vitro* conditions.

The production of HNE seems to be tightly connected to the oxidative burst of neutrophils. It was found that the formation of HNE correlated nicely with the production rate of superoxide anion at the site of inflammation (Figure 6). These data indicate that the mere number of neutrophils is a weak marker of inflammation. The oxidative burst and lipid peroxidation are more closely associated with the intensity of the inflammatory process as measured by the turnover rate of neutrophils.[13]

Taken together our results support the idea that HNE forms a part of an autocatalytic cycle of inflammation whereby neutrophils which immigrate into an inflammatory focus produce HNE as a consequence of the oxidative burst and HNE in turn may stimulate the ingress of further neutrophils.

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