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Humate-induced activation of human granulocytes

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Summary. Naturally occurring humic substances are particular chemical compounds which are found in humus. They bind to carbohydrates, amino acids and steroids by means of hydrogen bonds, covalent bonds and epsilon donor-acceptor complexes. Three specimens of low-molecular humic substances were tested (two naturally occurring humates and one synthetically prepared humate). They were all capable of stimulating certain functions of human neutrophils (PMN), such as the respiratory burst which results in the production of toxic oxygen compounds. This PMN stimulation can be demonstrated with the help of chemiluminescence, as well as by cytochemistry and with the electron microscope. The main product of the humate-induced PMN response is H_2O_2. There was no activation of neutrophilic chemokinesis or chemotaxis. It is suggested that the low-molecular humic substances originating from decaying organic material contain chemical structures which can act as signals to change dormant PMN into activated cells.

Key words: Humic substances – Humate – Human neutrophils – Respiratory burst – Chemiluminescence – Electron microscopy

Introduction

Naturally occurring humic substances (Berzelius 1893; Hoppe-Seyler 1889) are particular chemical compounds which are found in humus. They have a brown colour and are formed during the process known as humification from dead rotting organic material.

Humic acids arise from their precursors during the course of humification and, together with other non-humic substances, bind themselves to chemically inert humines which are finally incorporated into the process of carbonisation (Ziechmann 1980, 1988). In comparison with the humic acids themselves and with humines, the precursors of humic acids have a low molecular weight of about 1 kD. It is possible to reconstruct a model of their genesis, and it has been described mathematically by Kappeler and Ziechmann (1969) as a sequence of interrelated Markoff processes (Kingman 1969; Batschelet 1979). The synthesis of such a model humic substance is initiated by the autoxidation of multivalent phenols (Diebler et al. 1961; Ziechmann 1980), which appear naturally following the hydrolisation of lignins. As the result of the electrophilic attack on a displaceable electron by an epsilon-acceptor – usually molecular oxygen – highly reactive radicals are formed, which possess a single unpaired electron. The instability of such an oxygen radical is partly, but not completely, reduced by intramolecular mesomery. This leads to the appearance of, for instance, p-benzo-semiquinone anions as O- or C-radicals. As a result of the ever-increasing intramolecular mesomery, two C-radicals may become united by a C-C- bond, or a C- and an O-radical by an -O- bond.

Finally, the building up of side chains may be brought about by nucleophil substitution. In this way large molecular complexes can originate from the binding together of individual chemical building-blocks to produce a complex end structure (Ziechmann 1988; Thurmann and Malcolm 1983). The molecular bonds resulting from epsilon transfer between the individual molecules are further stabilised in this manner, and this produces intermolecular mesomery. Some molecules act as epsilon donors, and others as epsilon acceptors, thereby building up epsilon donor-acceptor complexes (Ziechmann 1988). That these processes also take place in naturally occurring humic substances can be demonstrated from the fact that they possess a high degree of electrical conductivity and act as weak semiconductors in the solid state (Lentz and Ziechmann 1967). The final stage consists of the appearance of carboxyl groups, which are brought into existence by the oxidation of CH_3- groups or by the opening of rings with subsequent oxidation (Ziechmann 1980).

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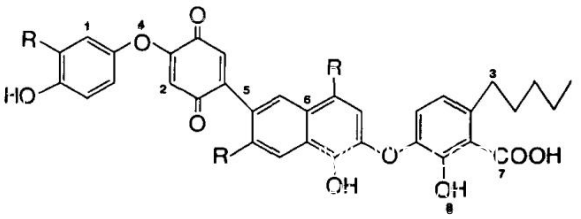


Fig. 1. Important structural characteristics of a model humic substance. *1.* aromatic ring; *2.* chinoid ring; *3.* aliphatic side-chain produced by breaking up of ring structure; *4.* O-bonds; *5.* C-bonds; *6.* C-C-bonds; *7.* carboxyl groups; *8.* phenolic -OH groups (R = 1–8)

Humic acids and humates therefore consist of numerous phenol and quinone rings (Fig. 1), brought together by -O-, -C- and -C-C- bonds to form complex building blocks which cannot be defined in classical chemical terms (Thurman and Malcolm 1983; Ziechmann 1988). These building blocks can bind themselves to amino acids and peptides, as well as to carbohydrates and steroids, by means of covalent bonding, hydrogen bonding or epsilon donor-acceptor complex bonding (Müller-Wegener 1982; Ziechmann 1988), bringing about an increase in entropy of the dead material up to a particular level. In this way humines are produced which lose their chemical reactivity pari passu with an increase in molecular weight.

These physicochemical properties of the low-molecular humic substances seem to be responsible for some reactions occurring in tissues (Jonas and Riede 1979, 1990; Riede et al. 1990; Kühnert 1989), such as antibacterial effects (Kühnert et al. 1989), antiviral effects (Kühnert et al. 1989) desmutagenic effects (Sato et al. 1986, 1987; Gichner et al. 1990) and also stimulating effects on oxidative phosphorylation in rat liver mitochondria (Visser 1987). To evaluate the possible role of humates in the inflammatory process (Lange et al. 1987), we stimulated human neutrophil granulocytes (PMN) with different natural and synthetically prepared humic substances and studied their reaction histochemically, under the electron microscope and with the help of chemiluminescence.

Material and methods

Humic substances. The following three preparations of humic substances (humates) have been available to us:

1. Unpurified natural raw humate obtained from Farberde (Kasseler Braun) with an average molecular weight of 15 kD (1.5–50 kD);
2. Purified natural humate (FL-70) obtained from Farberde (Kasseler Braun) (Mach 1980) with an average molecular weight of 3 kD (0.5 kD–5 kD);
3. Highly purified synthetic humate (HS-1500) (Seubert et al. 1989) with an average molecular weight of 1.5 kD (0.5 kD–2 kD).

PMN isolation. Human PMN (polymorphonuclear neutrophil granulocytes) were isolated from heparin-anticoagulated venous blood from healthy donors in a way that has already been described (Kapp et al. 1989, 1988), by Ficoll gradient centrifugation and three 30 s cycles of 0.2% NaCl treatment followed by the addition of an equal volume of 1.6% NaCl to lyse red blood cells. Cells were 98% PMN as judged by Pappenheim staining and more than 95% viable by trypan blue exclusion. They were used immediately for the experiments in order to avoid storage-associated alterations in the cytoskeleton (Palm et al. 1981).

Chemiluminescence. PMN (5×10^6 cells per ml) were placed in a solution of HBSS (Hank's balanced salt solution), buffered at pH 7.4 with HEPES (4-(2-Hydroxyethyl)-1-piperazine-ethanesulfonic acid), to which 2.2 ml of lucigenin and 1 mg/ml BSA had been added. Measurement of the lucigenin-dependent chemiluminescence was made after the addition of humate fractions and stimulation for 60 min at 37° C, as has previously been described (Kapp et al. 1985). The results were recorded as integral counts $\times 10^{-6}$.

Electron microscopy. In order to investigate the effect of the humic substances, PMN (5×10^6 cells per ml) were placed in microreaction chambers for examination under the transmission electron microscope, and in a concentration of 1×10^6 cells per ml in Leighton tubes (Costar-Tecnorama, Fernwald) for examination with the scanning electron microscope. Finally, PMN were treated with various concentrations of humate substances for 30 min at 37° C at pH 7.2, and either stimulated or allowed to remain unstimulated. Stimulation with phorbol myristate acetate (PMA) (10 ng/ml) served as a positive control. Following the relevant incubation time, the PMN were immediately fixed for transmission or scanning electron microscopy by a method already described (Zeck-Kapp et al. 1989), or further treated for the demonstration of H_2O_2 and then examined with the transmission electron microscope (Zeck-Kapp et al. 1989).

For examination with the scanning electron microscope, PMN were fixed in glutaraldehyde and then post-fixed in osmium tetroxide by a method already described (Zeck-Kapp et al. 1989). The specimens were dehydrated in an acetone series until a critical drying point had been reached, and then gold-sputtered and examined under a Jeol J-35 scanning electron microscope.

Histochemistry. The combined ultrastructural and histochemical demonstration of H_2O_2 production involved a method described by Briggs and his co-workers (1975). Briefly, after centrifugation, unfixed cells were preincubated for 10 min at 37° C in 0.1 M Tris-maleate with 7% sucrose, pH 7.5, containing 1 mM Aminotriazole (AT). The final incubation medium consisted of 0.1 M Tris-maleate with 7% sucrose, pH 7.5, 10 mM AT and 1 mM $CeCl_3$. PMN were incubated for 30 min at 37° C in this medium. Thereafter, PMN were washed in Tris-maleate buffer with 7% sucrose at 4° C and fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3, with 5% sucrose for 60 min at 4° C. In the cerium precipitation series, cells were subsequently washed in sodium cacodylate-buffer, pH 6.0, with 5% sucrose for 60 min at 4° C. The PMN were finally washed overnight in a 0.1 M sodium cacodylate buffer at pH 7.3 and 5% sucrose, and post-fixed for 60 min in 2% osmium tetroxide in the same buffer. Dehydration in ascending alcohols was followed by embedding in araldite. The ultrathin sections were routinely cut with a Reichert Ultratom 3, stained in 4% aqueous uranyl acetate and lead citrate and examined under a Zeiss 9 S-2 electron microscope.

Chemotaxis. Chemotaxis was examined in a Sykes-Moore chamber under an inverting microscope (Leitz Diavert) equipped for reflection contrast microscopy by a method that has already been reported (Keller 1983). The percentage of PMN that had migrated out of the total number of cells on each surface was determined (Keller 1983) with different concentrations of humate (HS-1500), three tests being carried out for each. An immunocomplex-activated specimen of human plasma served as a positive control medium (Keller et al. 1976).

Chemokinesis. The investigative routine used for chemotaxis is also appropriate for chemokinesis (Keller 1983). The motility of non-adherent, free-floating PMN was determined, and the proportion

Table 1. Lucigenin-dependent chemiluminescent (=CL) response due to various stimuli acting on PMN

Stimulus	CL response (Counts $\times 10^{-6}$)
Humate FL-70	
1000 µg/ml	74.3 ± 7.6[a]
100 µg/ml	300.2 ± 6.3
10µg/ml	234.1 ± 30.5
1µg/ml	9.0 ± 3.4
Background	4.4 ± 1.6
Phorbol myristate actetate (10 ng/ml)	871.8 ± 29.7

[a] The values correspond to the mean ± SE from 3 experiments on blood from 3 different donors (i.e. a total of 9 experiments)

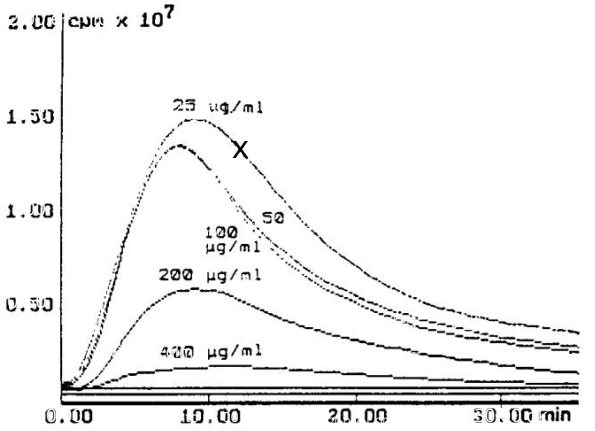
of PMN showing "crawling-like" movements (polarised cells) and the number of round (unpolarised) cells were counted in two different experiments with varying concentrations of humate (HS-1500).

A specimen of human plasma, to which a chemotactic hexapeptide (f-NPNTL) in a concentration of 10^{-9} M had been added, served as a control medium (Keller 1983). (f-NPNTL = N-formyl-L-norleucyl-L-leucyl-L-phenylalanyl-L-norleucyl-L-tyrosyl-L-lysin).

Results

Response to chemiluminescence

The unpurified natural fraction of humic substance, the purified natural fraction (FL-70) and the highly purified synthetic low-molecular fraction were all capable, in doses from 10 to 1000 µg/ml, of inducing a lucigenin-dependent chemiluminescent response. The maximum response was found with the purified humic substances in a concentration of about 100 µg/ml (Table 1). Up to this maximum value, the chemiluminescent response is dependent on the dose. Higher concentrations than 100 µg/ml of the humic substance produce a lower response (Table 1). None of the humic substance fractions investigated produced a chemiluminescent signal in the absence of granulocytes. As with phorbol myristate acetate (PMA 10 ng/ml), the arousal of the chemiluminescent response after stimulation with the three humic substances employed, produced a maximum response within 10 to 15 min after the addition of the humic substance, with a total kinetic of 60 min with the higher (100 µg/ml) and 120 min with the lower (10 µg/ml) concentration of the humic substances (Figs. 2, 3). The addition of superoxide dismutase, a specific scavenger of $O_2^{\cdot-}$, reduced the chemiluminescent response to the low-molecular humic substance fraction FL-70. In any case, catalase, which has a "scavenger specificity" for H_2O_2, significantly reduces the chemiluminescent response. On the other hand, D-mannitol, with a "scavenger specificity" for the hydroxyl radical OH, has a potentiating effect on the chemiluminescent response to humate (Table 2).



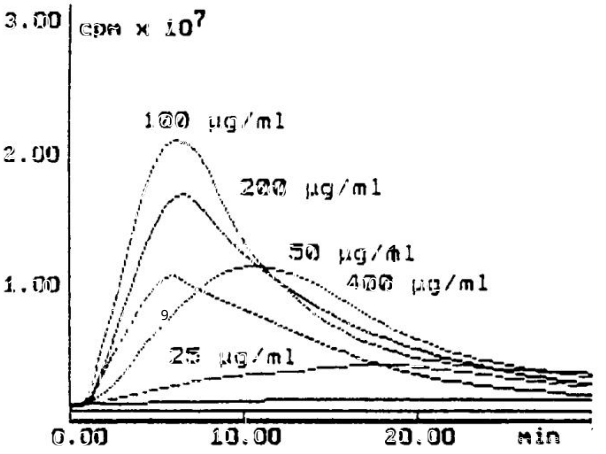


Fig. 2. Graphic representation of lucigenin-dependent chemiluminescent response following stimulation of human PMN with various concentrations of an unpurified natural humate from Farberde

Fig. 3. Graphic representation of lucigenin-dependent chemiluminescent response following stimulation of human PMN with various concentrations of a highly purified synthetic and low-molecular humate (HS-1500)

Table 2. Lucigenin-dependent chemiluminescent response following stimulation of PMN by humate (FL-70) under the influence of various inhibitors

Inhibitors	CL response (Counts $\times 10^{-6}$)
Humate (10 µg/ml)	
+ Medium	233.1 ± 35.6[a]
+ Superoxide dismutase (200 µg/ml)	144.6 ± 25.1
+ Catalase	99.3 ± 35.9
+ D-Mannitol (100 mM)	518.2 ± 91.9
Background	5.6 ± 1.7

[a] The values correspond to the mean ± SE from three experiments on blood from three different donors (i.e. a total of nine experiments)

Ultrastructural changes

Unstimulated PMN have a spherical appearance (Fig. 4a). Their surfaces are slightly folded, and this does not change during the incubation period. Under the

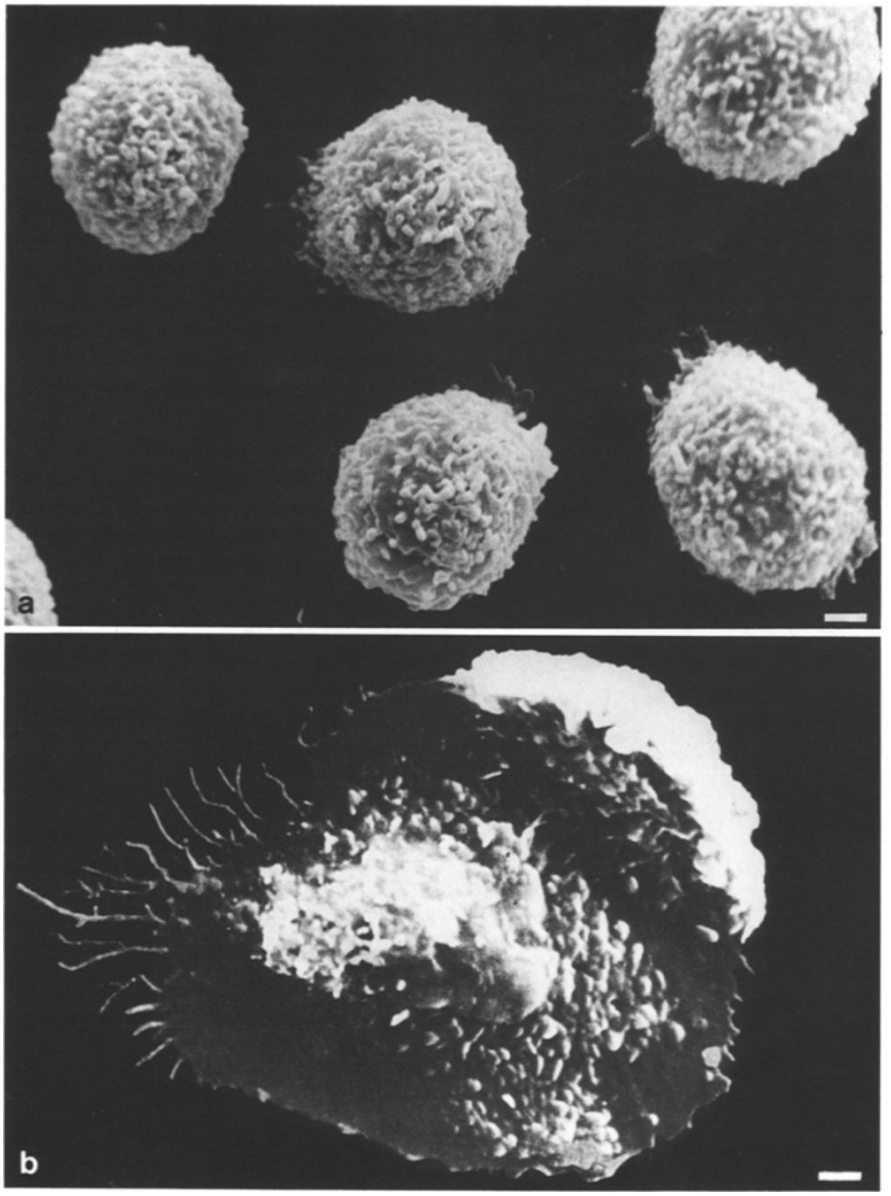
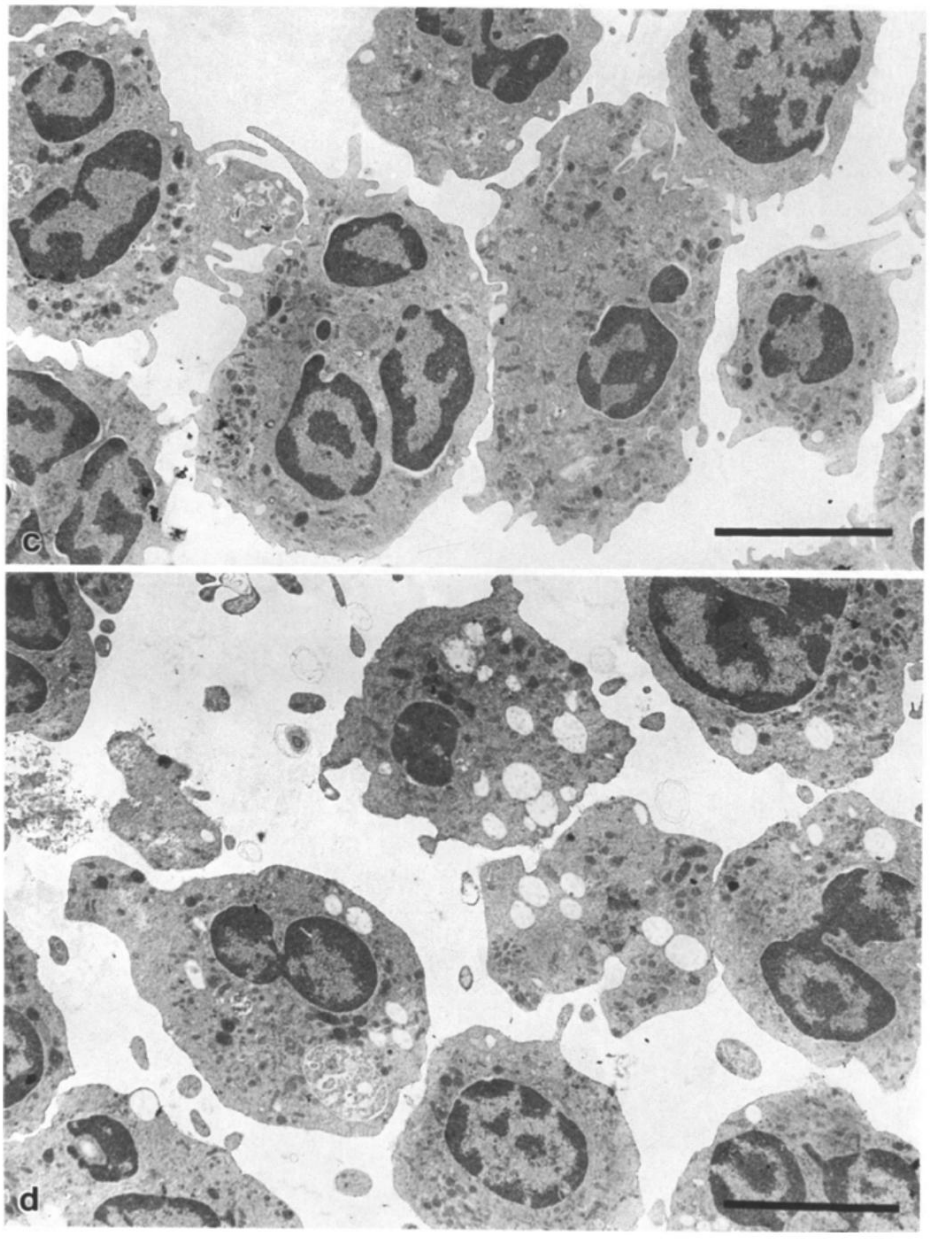


Fig. 4. a Unstimulated human PMN (15 min/37° C; SEM; Magn. ×2500). b Human PMN after stimulation with natural low-molecular humate (FL-70 100 µg/ml, 15 min/37° C; SEM; Magn. ×3800).

transmission electron microscope, their irregular multilobulated nuclei, surrounded by numbers of variously structured granules, are striking. In addition, several mitochondria and single collections of glycogen are found in the moderately dense cytoplasm (Fig. 4c). Following stimulation with 100 µg/ml of the humic substances FL-70 and HS-1500, characteristic changes have already appeared in the granulocytes: the cells aggregate (Fig. 5c), spread themselves over the plate and thus become considerably more flattened out, so that peripherally the granulocytes are limited by only a thin margin (Fig. 4b). As a result of the humate stimulation, one also sees electronlucent vacuoles lying immediately under the cell membrane (Fig. 4d). This signifies that both natural and synthetic humic substances possess the ability to activate PMN. In order to provide a comparable positive stimulus, PMA (10 ng/ml) was added. As with the humate, the spherical shape of the unstimulated granulocytes (Fig. 4a) disappears and the cells again become adherent to the plate, so that they disperse centrifugally and become markedly flattened (Fig. 5a). Under the transmission electron microscope one can observe the development of numerous pseudopodium-like projections of cytoplasm which arrange themselves around the periphery of the granulocyte like a wreath. In addition, one again sees numerous electron-lucent intracytoplasmic vacuolar structures of varying size (Fig. 5b). Stimulation of granulocytes with low-molecular humic substances or with PMA leads to the generation of H_2O_2, which is [illegible] and with the cytochemical investigations. The production of H_2O_2 during the activation of the granulocytes can be made visible under the transmission electron microscope as an electron-dense deposit of cerium perhydroxide. After stimulation for over 30 min with low-molecular humates (100 µg/ml), the outer side of the cell membrane and the luminal side of the intracellular electron-



c Unstimulated human PMN (15 min/37° C; TEM; Magn. ×6000). d Human PMN after stimulation with natural low-molecular humate (FL-70 100 µg/ml, 15 min/37° C; TEM; Magn. ×6000)

lucent vacuoles are covered with an electron-dense deposit of the cerium reaction products (Fig. 5d). The density of the specific deposit is entirely comparable with the reaction of the granulocytes following PMA stimulation (10 ng/ml) for 15 min. In the unstimulated cells, a deposit of cerium perhydroxide cannot be demonstrated. Therefore, no production of H_2O_2 can be detected.

Chemotaxis, chemokinesis

The addition of synthetic humate (HS-1500) in various concentrations produces no signs of chemotactic activity (Table 3). Neither could any be found with such low-molecular humate as that in which the PMN were chemokinetically stimulated (Table 4).

Discussion

It is clear from the present investigation that natural and synthetic humic substances stimulate certain functions of the PMN. Since this stimulation of the PMN can be demonstrated for both the unpurified and purified, and therefore for low-molecular humic substances, it may be assumed that the effect is associated with low-molecular humates or, at least, that it is not lost in the lower molecular fraction. PMN stimulation can be demonstrated with the help of chemiluminescence, as well as by cytochemistry and with the electron microscope. Measurement of the production of reactive oxygen metabolites by granulocytes using the chemiluminescent response is dose-dependent, and reaches a maximum at a humate concentration of 100 µg/ml. Higher concentrations than this lead to a reduced chemiluminescent re-

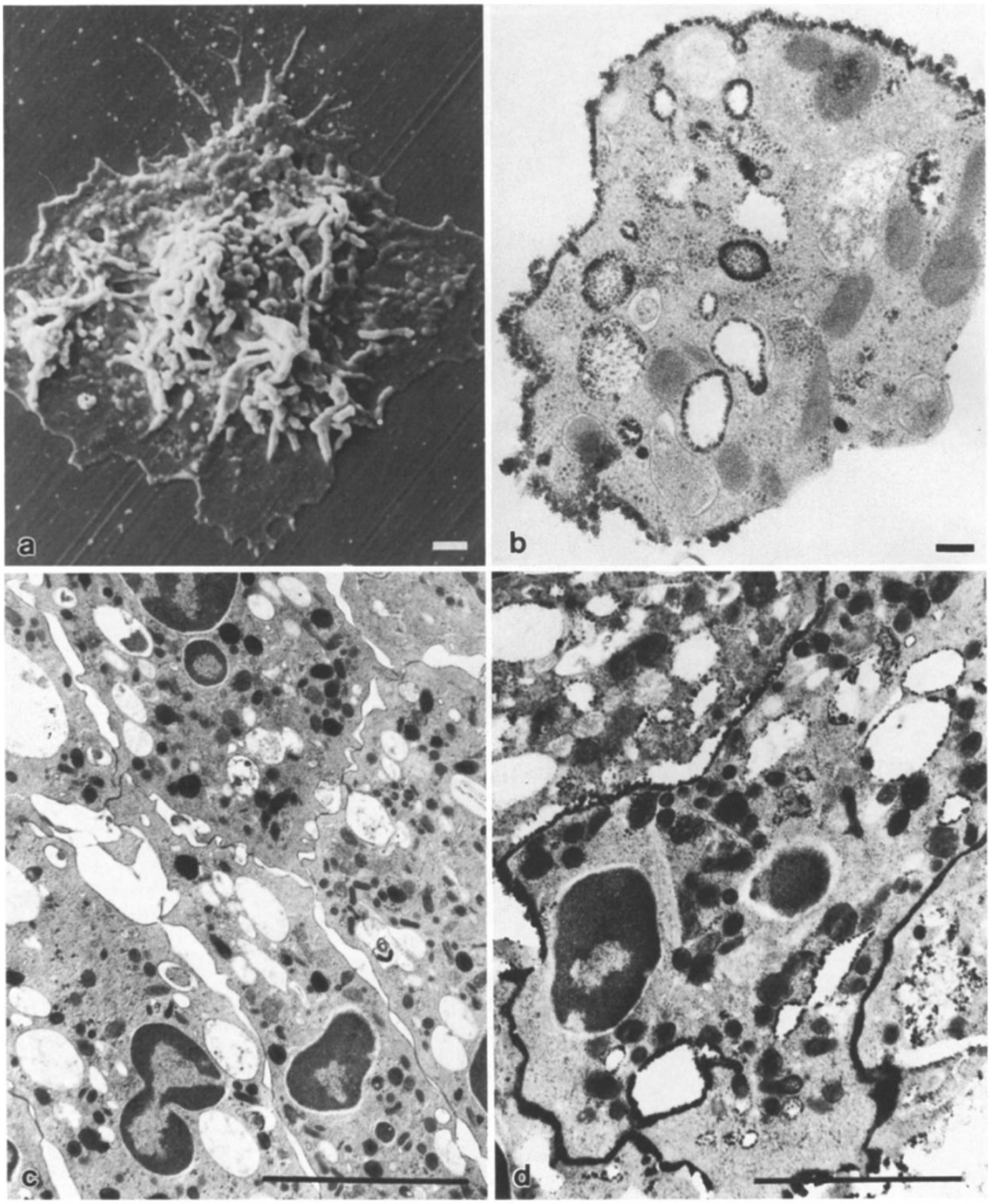


Fig. 5. a Human PMN after stimulation with PMA (10 ng/ml; 15 min/37° C; SEM; Magn. ×3000). b Ultrastructural demonstration of hydrogen peroxide production after stimulating PMN with PMA (10 ng/ml, 15 min/37° C; TEM; ×17000). c Aggregation of PMN following stimulation with humate (FL-70, 100 µg/ml; 15 min/37° C; TEM; Magn. ×4000). d Ultrastructural demonstration of hydrogen peroxide production after stimulating PMN with humate (FL-70, 100 µg/ml, 15 min/37° C; TEM; ×12000)

sponse on the part of the granulocytes. This may be because the use of humic substances with a pH 5–6 in high concentrations has a cytotoxic effect on the cell. Against this view, it can be claimed that the humate fractions were buffered at a pH 7.2, and showed ultrastructurally no signs of producing damage. On the other hand, it is also conceivable that humic substances, because they are potent epsilon acceptor-donor compounds, are themselves able to capture radicals and thus remove the oxygen-containing radicals which are produced. It is not possible to say, as a result of this investigation, whether the reduction of the effect with higher concentrations of humate is due to the oxygen radicals on the reactive surface of the cell being absorbed by the humate itself. It also follows that, in the absence of granulocytes, the humic substances employed produce no chemiluminescent signal and therefore reveal no source of reactive oxygen metabolites. Corresponding

Table 3. Percentages of migrated PMN per total PMN count during humate (HS-1500) stimulation

	$\bar{x}$	SD
1. Medium	0.19	± 0.1
2. Activated human plasma (20%)	22.82	± 12.01
3. 1 µg humate/ml	0.52	± 0.30
4. 10 µg humate/ml	0.46	± 0.20
5. 50 µg humate/ml	0.35	± 0.09
6. 100 µg humate/ml	0.23	± 0.10
7. 200 µg humate/ml	0.16	± 0.026

Means ± SD of three tests

Table 4. Change in cell shape of free floating PMN after humate (HS-1500) stimulation

	Round PMN in %		Polarised PMN in %	
	Mean	SD	Mean	SD
1. Medium	88.50	± 10.60	3.00	± 4.24
2. Medium ± FNLPNTL 10^{-9} M	9.00	± 1.41	89.50	± 6.36
3. Medium + humate 1 µg/ml	93.00	± 9.90	0.50	± 0.71
4. Medium + humate 10 µg/ml	86.00	± 16.90	1.00	± 1.42
5. Medium + humate 50 µg/ml	91.00	± 9.90	1.50	± 2.12
6. Medium + humate 100 µg/ml	86.50	± 10.60	0	± 0
7. Medium + humate 200 µg/ml	86.60	± 14.80	2.50	± 3.53

Percentage means ± SD of two tests

inhibition experiments on the production of reactive oxygen compounds with catalase have shown that H_2O_2 is obviously the main product of the humate-induced granulocytic response. Why D-mannitol should have a potentiating influence on the stimulation of granulocytes by humates is still not clear and requires further investigation. In comparison with other substances (Zeck-Kapp et al. 1989) which can activate granulocytes, it is apparent that the low-molecular humic substances obviously occupy an intermediate position between non-specific stimulation with phorbol esters and stimulation with cytokines such as GM-CSF and TNF (Kownatzki and Uhrich 1987; Kapp et al. 1988; Kownatzki et al. 1988; Zeck-Kapp et al. 1989). After being stimulated by humate, the granulocytes become active and adhere to the plate, obviously in readiness for chemotactic movement. However, they show no chemokinetic or chemotactic activity. As the main product of humate-induced activation, H_2O_2 appears on the surface of the cell. The corresponding histochemical reaction product reveals that activity of a membrane-bound oxidase, which is localised on the outer surface of the cell membrane (Badway and Karnovski 1980; Dewald and Baggioni 1979), has been induced. In comparison with the phorbol esters, the production of translucent vacuole-like inwardly-directed swellings of the cell membrane is a characteristic of granulocyte stimulation which is significantly more pronounced when brought about by humate. In contrast, however, to activation by phorbol esters, humate stimulates only partial functions such as the respiratory burst which results in the production of toxic oxygen compounds and has no mitogenic effects (Kaden and Riede unpublished work).

These findings indicate that both the natural and synthetic humic substances tested do release specific responses from the PMN particularly because thrombocytes do not respond to humates (Kanz and Riede unpublished work). An explanation for this lies in the fact that neutrophil granulocytes do not have capacity for differentiating between foreign and host antigens (Weiss 1989). For this they require support from the immune system in the form of antibodies, complement and cytokines. If normal host tissues are inappropriately identified as foreign, damaged or necrotic structures, the appropriate membrane receptors of the neutrophils will be engaged, eliciting the cells' destructive potential (Weiss 1989). It is even possible that low-molecular humic substances originating from decaying (in other words, necrotic) organic material contain chemical structures which can act as signals to change dormant neutrophil granulocytes into activated cells.

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References

Badway JA, Karnovsky ML (1980) Active oxygen species and the function of phagocytic leukocytes. Ann Rev Biochem 49:695–711

Batschelet E (1979) Introduction to mathematics for life scientist. 3rd edition Springer, Berlin Heidelberg New York, pp 427–429

Berzelius JJ (1893) Lehrbuch der Chemie, vol 8, pp 13–16. Berzelius JJ (ed) Arnoldische Buchhandlung, Dresden-Leipzig, pp 384–392

Briggs RT, Drath DB, Karnovsky ML, Karnovsky MJ (1975) Localization of NADH oxidase on the surface of human polymorphonuclear leukocytes by a new cytochemical method. J Cell Biol 67:566–586

Dewald B, Baggiolini M (1979) Subcellular localization of the superoxide forming enzyme in human neutrophils. J Clin Invest 63:21–37

Diebler H, Eigen M, Matthies P (1961) Kinetische Untersuchungen über die Bildung von p-Benzochinon aus Chinon und Hydrochinon in alkalischer Lösung. Z Naturforschung 16b:629–637

Gichner T, Badaev SA, Pospisil F, Veleminsky J (1990) Effects of humic acids, paraaminobenzoic acid, ascorbic acid on the N-nitrosation of the carbamate insecticide propoxur and on the mutagenicity of nitrosopropoxur. Mutat Res 299:37–41

Hoppe-Seyler F (1889) Über Huminsubstanzen, ihre Entstehung und ihre Eigenschaften. Hoppe-Seylers Z Physiol Chem 13:66–122

Jonas J, Riede UN (1979) Der Einfluß von Flavichromin (FL-70) auf das orthodontisch stimulierte Parodontium. Fortschr Kieferorth 40:70–78

Kapp A, Zeck-Kapp G, Danner M, Luger TA (1988) Human gran-

ulocyte-macrophage colony stimulating factor: A direct activator of human polymorphonuclear neutrophilic granulocytes. J Invest Dermatol 91:49–56

Kapp A, Danner M, Luger TA, Hauser C, Schöpf E (1987) Granulocyte-activating mediators (GRAM): II. Generation by human epidermal cells – Relation to GM-CSF. Arch Dermatol Res 279:470–492

Kapp A, Maly FE, Schöpf E (1985) Detection of complement activation in human serum using C5a-induced chemiluminescence of human granulocytes. Arch Dermatol Res 278:41–53

Kappler M, Ziechmann W (1969) Ein mathematisches Modell zur Beschreibung von Humifizierungsvorgängen. Brennstoff-Chemie 50:348–351

Keller HU, Gerber H, Hess MW, Cottier H (1976) Studies on the regulation of the neutrophil chemotactic response using a rapid and reliable method for measuring granulocytes. Agents and Actions 6:326–339

Keller HU (1983) Motility, cell shape and locomotion of neutrophil granulocytes. Cell Motility 3:47–60

Kingman JFC (1969) Markov population processes. J Appl Prob 6:1–18

Kownatzki E, Uhrich S (1987) Differential effects of nylon fibre adherence on the production of superoxide anion by human polymorphonuclear neutrophilic granulocytes stimulated with chemoattractants, ionophore A23187 and phorbol myristate acetate. Clin Exp Immunol 69:213–227

Kownatzki EI, Kapp A, Uhrich S (1988) Modulation of human neutrophilic granulocyte functions by recombinant human tumor necrosis factor and recombinant human lymphotoxin. Clin Exp Immunol 74:145–151

Kühnert M, Fuchs V, Golbs S (1989) Pharmacological and toxicological properties of humic acids and the way of their actions in veterinary medical therapy. Dtsch Tierärztl Wochenschr 96:3–10

Lange N, Golbs S, Kühnert M (1987) Basic research on immunological response of laboratory rats to humic acid. Arch Exp Vet Med 41:140–146

Lentz H, Ziechmann W (1967) Physikalisch-chemische Untersuchung an Huminstoffen. Brennstoff-Chemie 49:154–157

Mach W (1983) Verfahren zur Herstellung eines neuen biochemischen Wirkstoffes (FL-70). Eur Patent Office, Patent-Nr. EP 0035 586 B1. Courier Press, Leamington Spa, England

Mueller-Wegener U (1982) Wechselwirkingen von Huminstoffen mit Aminosäuren. Z Pflanz-Boden 145:411–420

Palm SL, Frucht LT, McCullough G (1981) Effects of temperature and duration of storage on granulocyte adhesion, spreading and ultrastructure. Lab Invest 45:82–88

Sato T, Ose Y, Nagase H, Hayase K (1987) Mechanism of the desmutagenic effect of humic acid. Mutat Res 176:199–204

Sato T, Ose Y, Nagase H (1986) Desmutagenic effect of humic acid. Mutat Res 162:173–178

Seubert B, Beilharz H, Fickert W, Günter J, Spitaler U (1988) Verfahren zur Herstellung von niedermolekularen Alkalihuminaten. Eur Patentoffice, Patent-Nr. EP 0313 718 A2. Courier Press, Leamington, England

Thurman EM, Malcolm RL (1983) Structural study of humic substances. In: Aquatic and terrestrial humic materials. Christman RF, Gjessing ET (eds). Ann Arbor Science, Ann-Arbor, pp 78–91

Visser SA (1987) Effects of humic substances on mitochondrial respiration and oxidative phosphorylation. Sci Total Environ 62:347–354

Weiss SJ (1989) Tissue destruction by neutrophils. N Engl J Med 320:365–376

Zeck-Kapp G, Kapp A, Riede UN (1989) Activation of human polymorphonuclear neutrophilic granulocytes by immuno-modulating cytokines. Immunbiology 179:44–55

Ziechmann W (1988) Evolution of structural models from consideration of physical and chemical properties. In: Frimmel FH, Christman RF (eds) Humic substances and their role in the environment. Wiley & Sons, New York, pp 113–132

Ziechmann W (1980) Huminstoffe. Verlag Chemie, Weinheim-Deerfield Beach, Florida

Ziechmann W, Kress B (1977) Über Elektronenzustände einer endoxydierten Huminsäure in wässriger Lösung. Geoderma 17:193–301

Ziechmann W (1988) Huminstoffe und ihre Wirkung in pedogenen Funktionseinheiten. Ökologie 5:49–66