Cellular models for the detection and evaluation of drugs that modulate human phagocyte activity

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Summary. Simple testing models have been developed for the evaluation of chemical or biological compounds that modulate the activity of human phagocytes. Human neutrophils from buffy coats of donor blood are used. They are stimulated with receptor agonists, and the effects of test compounds on exocytosis of different enzymes, the generation of superoxide (respiratory burst), and cytotoxicity are quantified. All assays are performed in microtiter plates and the responses are evaluated by multi-well photometry or fluorimetry. The models are apt to detect compounds acting on phagocytes as agonists or antagonists, signal transduction activators or inhibitors and primers of agonist responses, and to assess cytotoxic effects.

Key words. Phagocytes; cell activation; exocytosis; respiratory burst; activators; modulators.

Neutrophils and mononuclear phagocytes constitute the main cellular defence system of higher organisms against microbial infections. They collect in infected tissues, guided by chemotactic agents generated locally, where they phagocytose and usually kill the invaders. Upon chemotactic and phagocytic activation, the phagocytes release superoxide and H_2O_2, enzymes and other storage proteins, and a variety of bioactive lipids. Some of these products are required for the microbicidal function of the phagocytes [1, 2], and some induce inflammation and tissue damage, which are typical side effects of antimicrobial activity [14]. The balance between defence and inflammatory damage depends on the level of phagocyte activation, and therapeutic measures enhancing phagocyte activity to overcome infection and lowering it to tame inflammation are long-standing goals of the pharmaceutical industry. While neutrophil pathophysiology is largely restricted to infection and inflammation, mononuclear phagocytes – macrophages in particular – have much wider implications and are involved in processes as diverse as antigen presentation, atherosclerosis and possibly even tumor resistance. These considerations indicate the wide range of the potential influence of modulators of phagocyte activation.

Neutrophil activation. Mechanism of signal transduction

Neutrophil activation can be studied using chemotactic agonists or phagocytosable particles as the stimuli. Being soluble, receptor agonists have the advantage of acting instantaneously and uniformly (one ligand binding to one receptor) when added to a stirred neutrophil suspension. The interaction between particles and phagocytes, by contrast, is a progressive multi-receptor event with complex kinetics. Four chemotactic agonists, acting via separate receptors, have been characterized in recent years. They are the anaphylatoxin C5a formed upon complement activation via the classical or the alternative pathway [11], N-formyl-Met-Leu-Phe (fMLP) and other N-formylmethionyl peptides of bacterial origin [26], and two bioactive lipids, platelet-activating factor (PAF) and leukotriene B_4 (LTB_4) [5, 12, 16]. Unlike the peptide agonists, PAF and LTB_4 are produced by the (activated) neutrophils themselves, and can therefore function as amplifiers of the response elicited by the original stimulus [9]. A novel chemotactic peptide produced by human monocytes was recently described, and was termed NAF for neutrophil-activating factor or MDNCF for monocyte-derived neutrophil chemotactic factor [22, 33, 37]. All of the known chemotactic agonists induce shape changes, exocytosis and the respiratory burst. These functional responses depend on a rise of cytosolic free calcium ($[Ca^{2+}]_i$) and can be monitored in real time with appropriate instrumentation.

The mechanism of neutrophil activation, in particular the process of transduction of agonist signals, is largely unknown. Two elements are believed to be essential for this process, enhanced $[Ca^{2+}]_i$ and active protein kinase C. A role for protein kinase C is suggested by the fact that phorbol esters, e.g. phorbol myristate acetate (PMA), and permeant diacylglycerols (e.g. 1-oleoyl-2-acetyl-glycerol, OAG) elicit the respiratory burst [17, 25]. The requirement for calcium is indicated by the fact that in neutrophils depleted of intracellular storage-pool calcium (which are unable to rise $[Ca^{2+}]_i$ unless extracellular calcium is supplied) receptor agonists fail to induce exocytosis or the respiratory burst [13]. It is likely that upon stimulation with a receptor agonist, enhanced $[Ca^{2+}]_i$ is needed, together with diacylglycerol formation, to turn on proteinkinase C. A signal transduction sequence for the activation of human neutrophils, which can be proposed on the basis of information obtained in a number of laboratories, is illustrated schematically in figure 1. Studies on the mechanism of activation of the respiratory burst have shown that the activity of the NADPH-oxidase depends on ongoing agonist receptor binding [27], the interaction between the receptor and a GTP-binding protein [20] and the activation of phosphatidylinositol-specific phospholipase C [28]. This enzyme delivers the products which bring about the translocation of protein kinase C to the plasma membrane and its subsequent activation [15, 34]. Several results obtained under conditions indicated in the scheme support the proposed sequence. Signal transduction is prevented or inhibited by

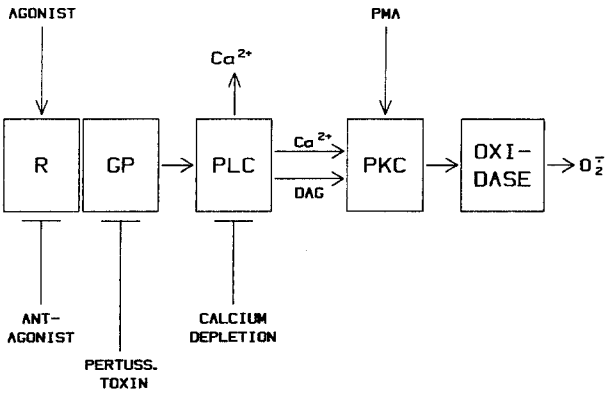

Figure 1. Interaction of an agonist with its receptor (R) coupled to a GTP-binding protein (GP) results in activation of the phosphatidylinositol-specific phospholipase C (PLC) and the formation of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate which in turn releases Ca^{2+} from intracellular stores. DAG and Ca^{2+} activate protein kinase C (PKC). The NADPH-oxidase (oxidase) forming superoxide (O_2^-) is activated as a consequence of the transduction process. Phorbol myristate acetate (PMA) can also activate the oxidase by acting directly on PKC.

receptor antagonists or by pretreatment of the cells with *B. pertussis* toxin, showing that receptor occupancy and functional GTP-binding proteins are necessary. As already mentioned, calcium-depleted cells do not transduce agonist signals eliciting exocytosis or the respiratory burst, indicating that a rise in $[Ca^{2+}]_i$ is required. The role of inositol-1,4,5-trisphosphate (IP_3) is suggested by the ability of exogenous IP_3 to induce the liberation of calcium in permeabilized neutrophils[24]. Finally the role of protein kinase C is suggested by the fact that the transduction sequence can be short-cutted by protein kinase C ligands like PMA.

We have recently studied the mechanism of human neutrophil activation by comparing the kinetics of the stimulus-dependent rise in $[Ca^{2+}]_i$ and H_2O_2 production during the respiratory burst.

Neutrophils loaded with quin-2 or fura-2 were stimulated at 37 °C in the presence and absence of extracellular calcium with fMLP, PAF or the calcium ionophore ionomycin. The experiments were performed in the stirred cuvette of a broad-band filter fluorimeter with a very fast response time, and the $[Ca^{2+}]_i$ changes induced by stimulation were recorded in real-time[32]. All three stimuli induced a rise in $[Ca^{2+}]_i$. The rise was faster and more extensive in the presence of extracellular calcium, indicating that calcium influx through the plasma membrane contributed to the change. A striking kinetic difference was observed between agonist- and ionophore-induced responses. The $[Ca^{2+}]_i$ rise induced by ionomycin was immediate while that induced by fMLP or PAF was preceded by a lag which increased with decreasing agonist concentration. These results suggested the involvement of a rate-limiting process controlling agonist-induced calcium release from internal stores and calcium influx across the plasma membrane[32]. Since IP_3 is considered to act as a second messenger liberating calcium from intracellular stores (see above), the lag could reflect the time required for the generation of threshold levels of IP_3. The lag was dependent on the agonist concentration and therefore on receptor occupancy, which most likely controls phospholipase C activity and thus the rate of IP_3 formation.

Signal transduction and activation can also be studied on the basis of the onset times of cellular responses like shape change, exocytosis or superoxide production. Such measurements must rely on very sensitive assay methods detecting the first appearance of products or behavioral changes. We therefore opted for a chemiluminescence measurement of the rate of H_2O_2 formation, which is a direct and highly sensitive assessment of NADPH-oxidase activity[35]. The chemiluminescence responses to different agonists varied considerably. When maximum effective stimulus concentrations were used, C5a yielded about 1/3 of the H_2O_2 produced upon stimulation with fMLP, while PAF and LTB_4 yielded only as little as 1/20 to 1/50 of that amount[36]. Nevertheless, the onset time of the response (2.4 s on average) was independent of the agonist used and its concentration. The kinetic similarities of the agonist responses suggest that signals acting on different receptors are transduced by a similar or even identical process[36]. The onset time of respiratory burst-associated chemiluminescence induced by ionomycin or PMA was at least 3–5 times longer than that observed upon stimulation with the agonists, indicating that receptor-dependent activation is due to a different and more efficient transduction process. Further experiments showed that the response to combinations of PMA and ionomycin, although more rapid, was still markedly slower than that elicited by agonists, showing that the transduction process initiated by the latter cannot be mimicked by concomitant protein kinase C activation and $[Ca^{2+}]_i$ rise.

The onset of the respiratory burst induced by agonists became even more rapid when the neutrophils were first treated with PMA. When PMA-pretreated cells were stimulated with fMLP, C5a, PAF or LTB_4 *before* a response to PMA becomes detectable, the onset time of H_2O_2-associated chemiluminescence production induced by the agonists was reduced to about half, i.e. to an average value of about 1 s. When, however, neutrophils that already produced H_2O_2 in response to PMA or OAG were stimulated with a receptor agonist, the ensuing burst of H_2O_2 production was apparently immediate and thus preceded the agonist-induced rise in $[Ca^{2+}]_i$. These results indicate that once protein kinase C is activated the NADPH-oxidase is turned on by the receptor agonist without appreciable changes in $[Ca^{2+}]_i$, and suggest that activation of protein kinase C could be a rate-limiting step in receptor-dependent neutrophil activation. The fact that agonists are much faster activators than PMA, and show synergism with PMA and other protein kinase C ligands suggests that receptor agonists initiate two signal transduction sequences. One sequence

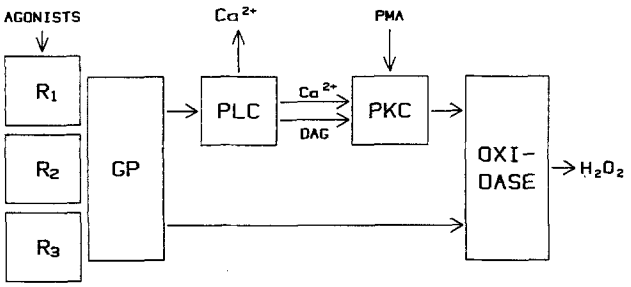

Figure 2. Expanded scheme of agonist-induced signal transduction in human neutrophils. R_1, R_2 and R_3 designate receptors specific for different agonists. They all become coupled to the same type of GP and induce similar signal transduction events. Two receptor agonist-dependent pathways are indicated that must work in concert to turn on the NADPH-oxidase. One involves PLC and PKC and depends on Ca^{2+}; the other appears to be Ca^{2+} and PKC independent. See text for details; abbreviations as in figure 1.

is calcium- and protein kinase C-dependent and rate-limiting, while the other is comparatively fast and appears to be calcium-independent. Both sequences are sensitive to *B. pertussis* toxin and are thus probably branching off downstream of G-protein. A scheme of this transduction system is shown in figure 2. Agonists acting on different receptors engage a common type of G-proteins. Activation of phospholipase C delivers diacylglycerol and leads (via IP_3) to a rise in $[Ca^{2+}]_i$ and to the activation of protein kinase C. This is not possible in calcium-depleted cells which do not respond to agonists unless PMA or other protein kinase C ligands are supplied. Two observations suggest that both sequences must be functional and must operate in concert for transducing receptor-dependent signals: a) Calcium depletion, which only affects the upper sequence of the scheme, blocks transduction; and b) the onset (i.e. transduction) time of agonist responses is shortened when the cells are pretreated with PMA or OAG, which act on protein kinase C and shortcut the rate-limiting sequence.

Mononuclear phagocyte responses

Human neutrophils are more readily obtained in large numbers than human mononuclear phagocytes, and for this reason most of the studies on phagocyte activation and signal transduction were performed with neutrophils. Centrifugal elutriation, however, yields relatively high numbers of reasonably pure human blood monocytes (about 10^8 cells of at least 90% purity per buffy-coat of a 400-ml blood unit [7]). Human macrophages, by contrast, cannot be obtained routinely, although bronchoalveolar or peritoneal lavage may yield satisfactory preparations of viable cells. We nevertheless consider human blood monocytes a better source: Upon culture for a number of days, monocytes mature into macrophages which remain viable for several weeks [7].

In a comparative study, we found that the respiratory burst responses (time course, rate and extent of superoxide and H_2O_2 production) of human neutrophils and monocytes to fMLP, C5a, PAF and LTB_4 are remarkably similar [5, 30]. This suggests that modulators of mononuclear phagocyte activation and recruitment can be detected on the basis of results obtained in primary screening systems with neutrophils. Monocytes and monocyte-derived macrophages, however, are indispensable for the study of inductive processes such as modulation by interferons [31] and other cytokines.

High-capacity assays for neutrophil activation

The method of choice for assessing neutrophil activation is to determine the formation of a product (like superoxide or H_2O_2) or the release of a component of the granules (an enzyme or other protein) in response to stimulation. Since our goal was to set up testing systems suitable for large-scale primary drug screening programs, we opted for a few simple and reliable assays that could be adapted to microtiter plates, and could be evaluated with multi-well detectors.

Exocytosis of elastase. Elastase, a neutral serine protease, is an exclusive component of the azurophil granules of human neutrophils [8]. Like other components of these organelles, e.g. myeloperoxidase and beta-glucuronidase, elastase is rapidly released upon stimulation by neutrophils pretreated with cytochalasin B. This pretreatment is required for the release of azurophil granule contents when the cells are stimulated with chemotactic agents, and enhances the general responsiveness of the cells [3]. Since elastase is active at neutral pH, the extent of release can be determined directly by the hydrolysis of a fluorogenic substrate that is included in the test mixture. The assay conditions can be adjusted to the experimental requirements. It should be noted, however, that the release response of the stimulated neutrophils is very rapid (60 s or less at room temperature [10]), and that a preincubation of the cells with the test compounds is thus recommended.

The conditions which were adopted for the evaluation of PAF antagonists [10] shall be summarized here as an example. 10^6 neutrophils per well are preincubated in 150 µl of PBS/BSA (PBS containing 2.5 mg/ml BSA since PAF is used as stimulus) in the presence of 125 nmol N-succinyl-Ala-Ala-Pro-Val-7-amido-4-methylcoumarin with or without test compound for 10 min. Cytochalasin B (1 µg in 50 µl PBS/BSA) is added, and after another 10 min the cells are stimulated with PAF (0.025 or 0.25 nmol in 50 µl PBS/BSA). Unstimulated controls (blank) receive 50 µl PBS/BSA in place of PAF. Starting of the reaction, i.e. addition of PAF to all 96 wells, requires about 1 min. Beginning 2 min after the start, repeated fluorescence readings are taken at 5- or 10-min intervals with a microplate fluorescence reader. The rate of fluorescence increase is a measure of the amount of elastase released. The whole test is performed at room temperature. For mixing after additions a Vortex Genie 2™ is suitable.

The specificity of the potential PAF antagonists is checked in the same test set-up by using several other agonists known to interact with different receptors (e.g. fMLP, C5a, LTB_4). Inhibition of elastase release induced by PAF but not by the other agonists indicates that the compound is specific for PAF. It shows in addition that it does not interfere with the measurement of elastase activity[10]. A control demonstrating that the test substance does not affect the detection system (in this case elastase activity) must always be included. A further control consists of incubating the neutrophils with the test compounds in the absence of a stimulus to determine possible stimulatory properties. It has been shown, for instance, that some antagonists have agonistic effects when used at high concentration.

When multiple concentrations of a stimulus are used or when the neutrophil-activating properties of different samples are studied, the reaction is started with the cytochalasin B-pretreated neutrophils to allow addition to all 96 wells within 1 min. Under these conditions, a batch of neutrophils (10^7 cells/ml) in the presence or absence of a test compound is incubated for 10 min with cytochalasin B (5 µg/ml), and 100 µl of this suspension is added to each well already containing elastase substrate and the appropriate stimulus in 150 µl PBS/BSA. Both procedures yield the same results as shown previously[10].

Exocytosis of beta-glucuronidase or N-acetyl-beta-glucosaminidase, two other constituents of the azurophil granules, can be determined alternatively. However, these activities cannot be measured in a one-step assay as in the case of elastase. In a first incubation, the cells are stimulated to release the granule contents, and then, following centrifugation and transfer of aliquots of the cell-free supernatants to a fresh microtiter plate, the enzyme activity is assayed with the respective substrate.

Stimulation of the neutrophils in the first microtiter plate is performed as described above for elastase release except that no enzyme substrate is added. After 5–10 min at room temperature the plate is centrifuged at 400 × g for 10 min. For the glycosidase assays, 25 µl of the cell-free supernatant are incubated with 25 µl 10 mM 4-methylumbelliferyl-beta-D-glucuronide in 0.1 M sodium acetate, pH 4.0, containing 0.1% Triton X-100 (beta-glucuronidase) or with 25 µl 10 mM 4-methylumbelliferyl-N-acetyl-beta-D-glucosamide in 0.1 M sodium citrate, pH 5.0, containing 0.1% Triton X-100 (N-acetyl-beta-glucosaminidase) for 60 min at 37 °C. In both cases the reaction is stopped by adding 200 µl glycine/EDTA buffer, pH 10.4 (0.1 M glycine, 10 mM EDTA) and the fluorescence of the liberated 4-methylumbelliferone is read with the microplate fluorescence reader. Alternatively, N-acetyl-beta-glucosaminidase can be determined spectrophotometrically with p-nitrophenyl-N-acetyl-beta-D-glucosaminide as substrate. Under these conditions 50 µl of the cell-free supernatants are incubated with 50 µl 7 mM substrate dissolved in 0.1 M sodium citrate buffer, pH 4.5, containing 0.2 M NaCl and 0.1% Triton X-100 for 60–120 min at room temperature. The reaction is terminated by addition of 100 µl glycine/EDTA buffer, pH 10.4, and the absorbance measured at 405 nm with a microplate reader.

Exocytosis of vitamin B_{12}-binding protein. Vitamin B_{12}-binding protein is most conveniently used as a marker for quantitating release from the specific granules. The neutrophils can be induced to release their specific granule content without concomitant release from the azurophil granules by stimulation with receptor agonists or with PMA in the absence of cytochalasin B. When cytochalasin B is present, agonist-induced release from the specific granules is enhanced.

Neutrophils (in the absence or presence of a test compound) are preincubated with or without cytochalasin B and are then stimulated as described above for release of glycosidases. After incubation for 10–30 min the microtiter plate is centrifuged and 25 µl of the cell-free supernatants are transferred to a new plate (with V-shaped bottom wells) for determination of the released vitamin B_{12}-binding protein. A blank consisting of 25 µl of PBS/BSA in place of supernatant must also be included. 50 µl of [^{57}Co]-labeled vitamin B_{12} (cyano[^{57}Co]cobalamin, 180–300 µCi/µg diluted 1:50 with 0.1 M potassium phosphate buffer, pH 7.5) are then added and the plate is kept in the dark (at room temperature with occasional mixing) for 30 min. Free and bound vitamin B_{12} are separated by adding 100 µl of a BSA-coated charcoal suspension (mixture of equal volumes of a 1% aqueous solution of BSA and a 5% suspension of Norit A charcoal). After another 15 min in the dark and occasional mixing the plate is centrifuged (1400 × g, 15 min). 100 µl of the clear supernatant are removed, and the radioactivity is measured in a gamma-counter. Total radioactivity corresponding to 50 pg vitamin B_{12} is determined separately in 50 µl of the diluted cyano[^{57}Co]cobalamin solution.

Respiratory burst. H_2O_2 formation can be used as a measure of the respiratory burst activity of stimulated neutrophils and mononuclear phagocytes. The colorimetric assay of Pick and Keisari[23], which is based on the horseradish peroxidase-mediated oxidation of phenol red by H_2O_2, was adopted. The formation of the phenol red oxidation product was quantified by the decrease in absorbance at 550 nm (modification by Collins-Boyce and Matteo, personal communication). The microtiter plate assay is set up as follows: To 10^6 neutrophils in 50 µl PBS/BSA are added 25 µl test substance (or PBS/BSA) and 100 µl of a reagent mixure containing 4.5 µg phenol red, 10 U horseradish peroxidase and 9 µg superoxide dismutase in PBS/BSA, pH 7.4. Where indicated 25 µl of cytochalasin B (40 µg/ml) or PBS/BSA are added and the mixtures are preincubated for 10 min at 37 °C in an incubator. The respiratory burst is initiated by addition of the appropriate stimulus in 25 µl PBS/BSA, and after another 15 min at 37 °C the reaction is stopped with 25 µl 1N NaOH. Absorbance is then measured in a microplate

reader using a 550 nm interference filter. When a receptor agonist (fMLP, C5a) is used as stimulus, the cells are pretreated with cytochalasin B to obtain higher rates of H_2O_2 production, whereas in the case of PMA cytochalasin B has no enhancing effect. An H_2O_2 standard curve extending from 0.5 to 10 nmol/well has to be included in each experiment. In additional controls it has to be shown that the test substances do not interfere with the H_2O_2 detection system.

Cytotoxicity test. Assay for the release of lactate dehydrogenase (LDH). Possible cytotoxic effects of the test compounds are evaluated by measuring release of the cytosolic marker LDH. This enzyme is dertermined in the cell-free supernatants obtained following stimulation of the neutrophils in the presence of substances as described above for release of glycosidases. Supernatants obtained from the one-step assay for release of elastase can also be used. LDH activity is assayed according to Bergmeyer and Bernt[6] in 50 µl of the cell-free supernatants as described by Dewald and Baggiolini[10].

Examples of application

The elastase release assay has been used in our laboratory in a screening for modulators of neutrophil activity produced by mononuclear phagocytes and lymphocytes from human blood, and was instrumental in the recent discovery of a neutrophil-activating factor (NAF) released by stimulated human monocytes. NAF was detected in the conditioned media of human monocytes which were cultured for 12–48 h in the presence of *E. coli* lipopolysaccharide (LPS) or lectins, and was identified as a novel peptide upon purification and amino acid sequencing[22,33]. Large numbers of samples from conditioned media and chromatography separations could be analyzed in a short time using the assay mode for comparing activators, where the test is started with cytochalasin B-treated neutrophils (see description of the assay). Figure 3 shows the release of elastase by neutrophils exposed to increasing concentrations of NAF. A linear relation was obtained by plotting the concentration of the activator on a logarithmic scale. Since the responsiveness of the neutrophils may differ from donor to donor, it is advisable to test complete sets of samples (e.g., all fractions from a chromatography experiment) in parallel with the same neutrophil preparation. It is also advisable to include a standard sample of the activator studied in each test series. Standardization on the basis of an unrelated stimulus (e.g., PAF or fMLP) is not reliable since the neutrophil responsiveness to different stimuli does not necessarily show parallel variations.

The elastase release assay was also very useful for the evaluation of PAF antagonists. These results are presented in figure 4 and table 1. For this purpose the neutrophils were preincubated with the test compounds in the microtiter plate, and the reaction was started by addition of the stimulus. As an example, 4 substances at 5 concentrations in quadruplicate, or 10 substances at 4 concentrations in duplicate could be tested simultaneously in one plate. Release of elastase induced in neutrophils by receptor agonists other than PAF was not inhibited indicating that these compounds acted specifically at the level of the PAF receptor. In addition, the test system was also suitable for obtaining information on the type of antagonism of selected compounds[10].

A different class of substances that inhibit elastase release by stimulated neutrophils is represented by wortmannin[4]. Table 2 shows that exocytosis induced by four dif-

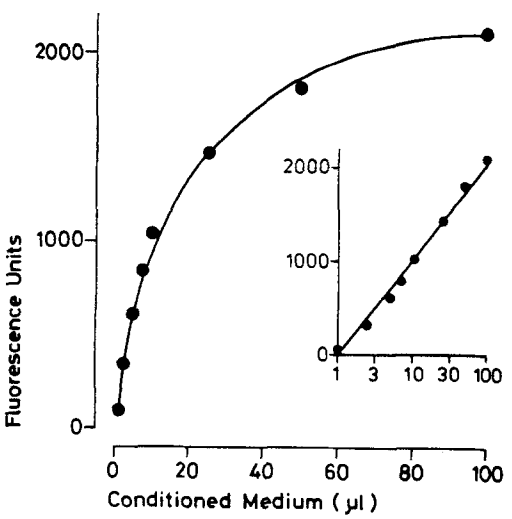

Figure 3. NAF-induced elastase release by cytochalasin B-treated human neutrophils. Dependence on the amount of NAF-containing conditioned medium added. 5×10^6 mononuclear cells were cultured for 24 h in 1 ml of medium containing 100 ng LPS. Inset: NAF plotted on a log scale. Reproduced from Peveri et al.[22] with permission.

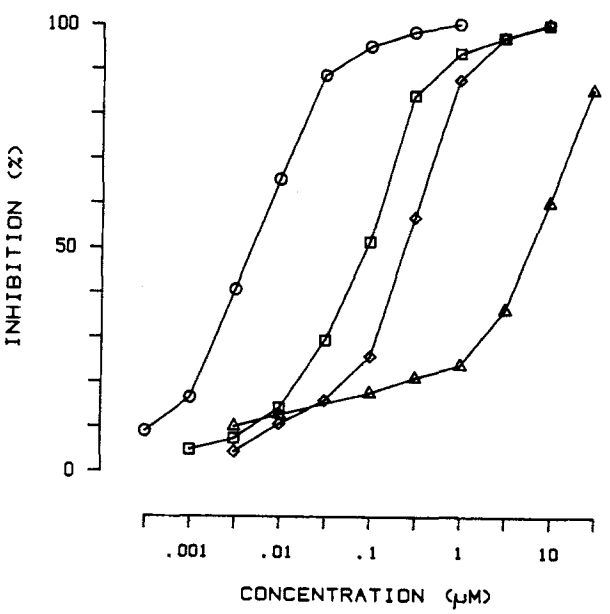

Figure 4. Effect of PAF antagonists on PAF-induced elastase release by human neutrophils. The antagonists were: Ro 19-3704 (○), BN 52021 (□), CV-3988 (◇) and 48740 RP (△). PAF was used at 0.1 µM. Reproduced from Dewald and Baggiolini[10] with permission.

Table 1. Inhibition of PAF-induced elastase release by PAF antagonists

Antagonist	IC_{50} (nM) PAF 0.1 μM	(n)	PAF 1.0 μM	(n)
Ro 19-3704	9 ± 6	(12)	174 ± 27	(6)
BN 52021	125 ± 60	(7)	228 ± 94	(6)
CV-3988	270 ± 147	(5)	4910 ± 614	(4)
Kadsurenone	438 ± 233	(5)	2520 ± 1200	(3)
48740 RP	6400 ± 1400	(6)	> 10,000	(6)

The IC_{50} values were obtained from experiments as shown in fig. 4. PAF was used at 0.1 and 1.0 μM. Values represent means ± SD with the number of experiments using different neutrophil preparations in brackets. Reproduced from Dewald and Baggiolini[10] with permission.

Table 2 . Effect of wortmannin derivatives on agonist-induced elastase release by human neutrophils

Stimulus	(μM)	IC_{50} (nM) WT	HWT	DAWT	iDAWT	(n)
fMLP	(0.01)	98 ± 68	96 ± 70	101 ± 55	>10,000	(4)
	(0.1)	340 ± 207	413 ± 274	295 ± 198	>10,000	(3)
C5a	(0.0001)	141 ± 38	54 ± 18	100*	>10,000	(4)
	(0.001)	476 ± 123	398 ± 292	510*	>10,000	(4)
PAF	(0.1)	90 ± 65	81 ± 56	74 ± 23	>10,000	(3)
	(1.0)	400 ± 227	323 ± 210	197 ± 38	>10,000	(3)
LTB_4	(0.01)	209 ± 103	127 ± 78	60**	>10,000	(5)
	(0.1)	454 ± 88	308 ± 63	640**	>10.000	(5)

Mean values ±SD from experiments performed with n different neutrophil preparations. * Average from 2 experiments; ** Single experiment. WT, wortmannin; HWT, 17-hydroxywortmannin; DAWT, desacetoxywortmannin; iDAWT, inactive desacetoxywortmannin (compound 11 in: Baggiolini et al.[4]).

ferent agonists, i.e. fMLP, C5a, PAF and LTB_4, was similarly affected by wortmannin and two of its derivatives suggesting that they interfere with signal transduction rather than receptor interaction.

These compounds are also potent inhibitors of the respiratory burst induced by receptor agonists, but have no effect when the burst is elicited by protein kinase C activators such as PMA. These results, obtained with the microplate assay for determining H_2O_2 formation by stimulated neutrophils, are shown in figure 5.

Profiles of biological activity detectable with the present testing system

A basic advantage of the testing system proposed lies in the use of human neutrophils, which obviates the problems of species specificity and predictability encountered when using animals, animal cells or cell lines. The tests mainly detect two types of activities: a) neutrophil activation, and b) modulation (decrease or enhancement) of neutrophil activity induced by a given stimulus.

Neutrophil-activating agents will elicit neutrophil responses directly (i.e., in the absence of a stimulus or other effector molecule). Such compounds are generally expected to induce more than one effect, and the detection of a positive response, like for instance elastase release, should automatically lead the investigator to the analysis

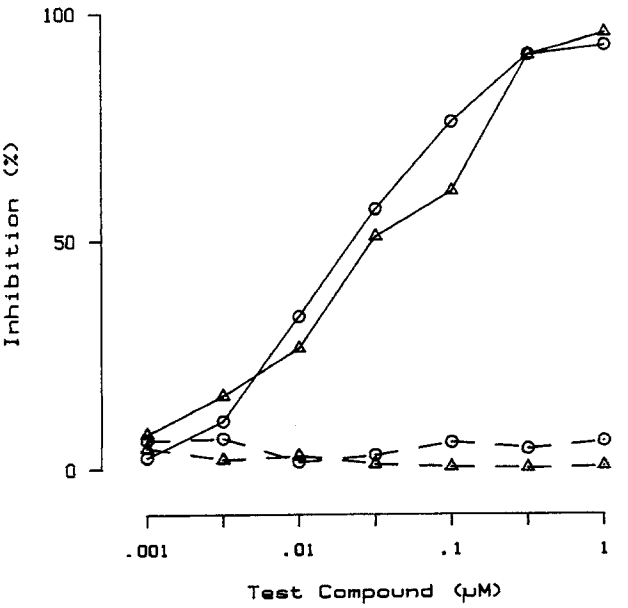

Figure 5. Effect of wortmannin derivatives on the respiratory burst. Human neutrophils were stimulated with 0.1 μM fMLP (—) or 0.03 μM PMA (----) in the presence of wortmannin (△) or 17-hydroxywortmannin (○).

release assay, as already mentioned, and was subsequently shown to behave like a chemotactic agonist, inducing in addition a rise in $[Ca^{2+}]_i$, shape changes, and the formation of superoxide and H_2O_2[22,29]. The responses to NAF were rapid, transient and *B. pertussis* toxin-sensitive, which is characteristic for receptor-mediated events, and could thus be readily differentiated from the effects of ionophore- and protein kinase C-type activators. Desensitization and cross-desensitization experiments showed in addition that NAF acts via a selective receptor, different from those of known neutrophil agonists[22].

Modulators of neutrophil activity comprise two main classes of compounds: Agents with a *priming or enhancing effect*, which render the cells more sensitive or responsive to stimulation, and *inhibitors* of different kinds which prevent, decrease or antagonize the effects of stimulation. The testing procedure is similar for both classes of compounds. As described above for the PAF antagonists, the test compounds are preincubated with the neutrophils, and a response is then elicited with the desired stimulus. PAF is a good example for a priming agent. PAF is a very weak inducer of the respiratory burst[36], but has a strong enhancing effect on the respiratory burst response elicited by chemotactic peptides, i.e., fMLP, C5a and NAF[5,9,22]. The priming effect is not apparent in the elastase release assay, where PAF itself is a potent stimulus of exocytosis[5].

We have presented here two classes of neutrophil inhibitors, receptor antagonists and signal transduction inhibitors. Different types of interventions can in fact pre-

or inhibition of effector functions like exocytosis or superoxide formation. Experimentally, it is not difficult to discriminate between these possibilities, and, on the other hand, to distinguish between selective inhibition of a cellular function and cytotoxicity[10].

When searching for potential drugs affecting the neutrophil one should realize that in many cases they will also affect the mononuclear phagocytes, i.e., the monocytes and the macrophages. Macrophages derive from the monocytes and persist for long periods of time in different tissue locations performing an impressively large variety of functions[19]. Both mononuclear phagocytes respond to most stimuli which activate neutrophils (e.g. fMLP, C5a, PAF, PMA and phagocytosable particles of various kinds)[5, 30], are primed, like neutrophils, by PAF and protein kinase C activators[5, 30], and are inhibited by wortmannins[4]. It appears justified, therefore, to use the readily available neutrophils to search for molecules acting on mononuclear phagocytes.

Even more distant goals may be pursued with the screening system described: The search for antagonists of mediators which stimulate other cells in addition to neutrophils. In view of its reliability and easy handling, the elastase release assay can be adopted for the search of mediator antagonists with wider pathophysiological implications. This is the case in particular for PAF, which stimulates a large variety of cells and is considered as a key mediator in asthmatic disorders[21]. But it also applies to C5a, involved in anaphylactic reactions, and possibly to LTB_4, the effects of which beyond the neutrophil are still being explored.

The rationale behind the search for phagocyte activators and/or modulators

In view of their ability to induce the release of microbicidal products, phagocyte activators could be beneficial in the therapy of conditions of impaired host resistance. Phagocyte priming agents are at present more appealing because they enhance the responsiveness to stimulation rather than eliciting a response directly. Their potential use remains to be established, but the ongoing research with colony-stimulating factors, in particular GM-CSF, as enhancers of neutrophil-dependent microbicidal activity appears very promising[18]. The possible use of NAF, the newly-discovered neutrophil activator, will have to be explored when it becomes available in larger quantities. It will be important to find out whether NAF at low concentrations has priming rather than activating properties.

The other face of the coin, phagocyte inhibition, has been a long-standing goal of inflammation research. Inflammatory processes are sustained by activated phagocytes which release phlogogenic and tissue-damaging products, and which ensure a steady influx of inflammatory cells into the affected sites. These self-perpetuating processes could be interrupted by a temporary (and reversible) deactivation of phagocytes. Up to now, the search for compounds fulfilling this task has been deceiving. Better knowledge of the mechanisms of phagocyte activation and a more efficient screening methodology, as presented in this account, constitute a promising starting point toward new classes of anti-inflammatory drugs.

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Development of DNA probes for cytotoxin and enterotoxin genes in enteric bacteria

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Summary. DNA probes to identify the genes encoding toxins in enteric bacteria have been developed. Use of these probes reduces the number of animals required for toxicity testing, as suspect bacteria can be directly tested for the presence of toxin. We have augmented the gene probes available by developing probes against the *Escherichia coli* enterotoxin LTII and shiga toxin from *Shigella dysenteriae* 1.
The LTII gene from *E. coli* 357900 was identified and characterised and a suitable internal probe was obtained. The LTII gene was found not to be common among enterobacteriae from various geographical locations. Isolates predominately of animal origin from Nigeria and Thailand hybridized with the probe.
The shiga toxin gene was isolated from *S. dysenteriae* 1 by a combination of in vivo and in vitro methods. An internal probe was identified and used against different serogroups of *Shigella* and *E. coli* isolates. The probe was found to hybridize with *S. dysenteriae* 1 isolates and also some *S. flexneri* and *S. sonnei* strains. Representatives were tested for toxin production and found to produce toxin at low levels.
Key words. DNA probes; cytotoxin and enterotoxin genes; *Escherichia coli; Shigella* spp.

Introduction

Diarrhoea, the rapid loss of water and salts from body via the intestinal tract, is the most important infectious disease of our time: there are some 10^9 cases per year worldwide and several million deaths. Although the severe diarrhoea is mostly seen in developing countries, where most deaths due to diarrhoea occur in infants of two years and below, a high proportion of individuals in developed countries experience at least one episode of diarrhoea per year (often when travelling, hence 'travellers diarrhoea') and infants in day centres generally suffer multiple episodes per year. In addition to the human suffering caused by diarrhoea, large numbers of