Granulocyte-macrophage colony-stimulating factor can stimulate macrophage proliferation via persistent activation of Na^+/H^+ antiport

Evidence for two distinct roles for Na^+/H^+ antiport activation

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Macrophages respond to a variety of extracellular stimuli which can modulate the proliferation, development, activation and functional activity of these cells. The effects of two such agents, granulocyte-macrophage colony-stimulating factor (GM-CSF, which stimulates proliferation) and platelet-activating factor (PAF, which stimulates chemotaxis and bactericidal activity), on cellular signal transduction mechanisms were compared. PAF can stimulate inositol lipid hydrolysis leading to Ca^{2+} mobilization. GM-CSF on the other hand has no effect on these events. Both agonists do, however, share an ability to activate an amiloride-sensitive Na^+/H^+ antiport and, furthermore, amiloride analogues are shown to inhibit the proliferative effects of GM-CSF on these cells. Long-term incubations with either PAF or GM-CSF demonstrate that it is only those cells pretreated with the latter which show a persistent activation of the antiport together with a sustained increase in intracellular pH. PAF-treated cells exhibit only a transitory increase in antiport activity, their intracellular pH levels returning to resting levels in spite of the continuous presence of the agonist in the medium. These effects of GM-CSF and PAF on Na^+/H^+ exchange are observed in both bicarbonate-free and bicarbonate-containing medium. These results lead us to suggest that the Na^+/H^+ antiport has a role in macrophage proliferation and in the regulation of intracellular pH during the oxidative burst stimulated by PAF and other agonists, and that differential mechanisms whereby this antiport is regulated exist in macrophages.

INTRODUCTION

Mononuclear phagocytes, or macrophages, have a wide variety of functions including cell-mediated cytotoxicity, phagocytosis of dead cells and particulate matter, and in antigen presentation (North, 1978; Babior, 1984; Adams & Hamilton, 1984). Because of their finite lifespan, constant replacement of peripheral blood and tissue macrophages is required. This is achieved by their replacement from pluripotent haemopoietic stem cells which differentiate and develop to give all of the mature blood cells found in the blood and tissues (see Metcalf, 1984). The control of this process of haemopoiesis is thought to be mediated by specific haemopoietic growth factors which can control the survival, proliferation and development of immature progenitor cells to form the appropriate mature cell.

Extensive studies have shown that several distinct growth factors can promote macrophage proliferation; these include interleukin 3 (IL-3), colony-stimulating factor 1 (CSF-1) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Metcalf, 1970; Whetton

et al., 1986; Hamilton *et al.*, 1986, 1988). Although these growth factors can undoubtedly stimulate myeloid cell production *in vivo* and *in vitro*, little is known about the molecular mechanisms whereby they stimulate cellular proliferation and development. It has, however, been clearly established that leukaemic transformation is associated with either independence from or an altered response to haemopoietic growth factors (Lang *et al.*, 1985; Lowenberg *et al.*, 1988). For these reasons it is necessary to establish the events elicited by these growth factors in normal haemopoietic cells and the possible effects of leukaemic transformation on these events.

Unlike the haemopoietic system, other cell types have been extensively employed to establish the basis of growth-factor-mediated cell proliferation and the ways in which oncogenic transformation may negate the controlling influence of these growth factors (Hunter & Cooper, 1985; Rozengurt, 1986; Weinstein *et al.*, 1988). In such studies, a number of signal transduction mechanisms have been identified which are associated with growth-factor-stimulated proliferation. These include increases in phosphoinositide turnover, Ca^{2+} mobiliz-

Abbreviations used: GM-CSF, granulocyte-macrophage colony-stimulating factor; IL-3, interleukin 3; PAF, platelet-activating factor; M-CSF (CSF-1), macrophage colony-stimulating factor (Colony stimulating factor 1); pH_i, intracellular pH; NBMD macrophages, normal bone-marrow-derived macrophages; BCECF, bis(carboxyethyl)carboxyfluorescein; 5-MNIA, 5-(N-methyl-N-isobutyl)amiloride; ED₅₀, dose at which 50% of maximal effect was observed (effective dose); ID₅₀, dose at which 50% inhibition of effect was observed (inhibitory dose).

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ation and ion fluxes, in particular the exchange of H^+ for Na^+ by the activation of an Na^+/H^+ antiport resulting in cytoplasmic alkalinization (Rozenfurt, 1986). In macrophages, agonists (e.g. platelet activating factor, PAF) which stimulate inositol lipid hydrolysis and elevated Ca^{2+} levels have been demonstrated to activate a variety of functional effects, such as chemotaxis and reactive oxygen intermediate production (Huang *et al.*, 1988), but they are not mitogenic.

Here we have investigated the effects of PAF and GM-CSF on macrophage Na^+/H^+ antiport activity and intracellular pH (pH_i), and show that these agents can both activate Na^+/H^+ exchange, but by using distinct mechanisms.

MATERIALS AND METHODS

Preparation of macrophages derived from murine normal bone marrow

Macrophages derived from normal murine bone marrow (NBMD macrophages) were prepared as previously described (Whetton *et al.*, 1986), with the exception that, in order to grow non-adherent macrophages, 25 ml or 50 ml vol. of the normal bone marrow cell suspension mixture (Whetton *et al.*, 1986) were placed in sterile teflon bags (Cooper *et al.*, 1984). These preparations gave rise to > 95% pure macrophage cultures.

Analysis of inositol phosphates

Macrophages were isotopically labelled with [3H]inositol as previously described (Whetton *et al.*, 1986). This labelling procedure leads to the incorporation of substantial amounts of [3H]inositol into various cellular metabolites with no apparent loss of cellular viability.

Measurement of [3H]inositol phosphates was performed as previously described for NBMD macrophages (Whetton *et al.*, 1986; Huang *et al.*, 1988). The total $InsP$ fractions consisted of column eluates containing $InsP$, $InsP_2$ and $InsP_3$.

Measurement of [3H]thymidine incorporation

[3H]Thymidine incorporation into macrophages was performed as a measure of cellular proliferation as described by Whetton *et al.* (1986), with one modification. Unless otherwise stated, Hepes (10 mM)-buffered, bicarbonate-free Fischer's medium was employed in the experiments described.

Intracellular Ca^{2+} measurements

Intracellular Ca^{2+} concentrations were determined using the Ca^{2+} -sensitive fluorescent probe quin-2 (Tsien *et al.*, 1982). Macrophages grown in teflon bags were harvested, washed twice in Fischer's medium and resuspended in 0.14 M-NaCl/2.68 mM-KCl/8.10 mM- Na_2HPO_4 /1.47 mM- KH_2PO_4 /10 mM-glucose/1 mM- $CaCl_2$ /0.001% (w/v) bovine serum albumin BSA (buffer A). The cells were then incubated with quin-2/AM (final concn. 40 μM) at 37 °C for 30 min. After this time, the cells were washed twice in buffer A and resuspended to a final concentration of between 10^6 and 10^7 cells/ml. Fluorimetric determinations were made in buffer A at 37 °C using a Perkin-Elmer LS5 fluorescence spectrometer, essentially as described by Whetton *et al.* (1988a).

Intracellular pH measurements

pH_i was determined using the fluorescent pH-sensitive

probe bis(carboxyethyl)carboxyfluorescein (BCECF) (Tsien *et al.*, 1982). For these experiments, macrophages were grown in teflon bags for 7 days, then prepared for assays of pH_i as described by Whetton *et al.* (1988b), except that the cells were suspended either in buffer A (see above) or in a bicarbonate-containing buffer as detailed by Ganz *et al.* (1989). After loading the NBMD macrophages with BCECF, the cells were washed twice in the above buffer and resuspended to a final concentration of between 5×10^5 and 1×10^6 cells/ml, and taken for fluorimetric pH_i assays.

Where cells were preincubated with agonists prior to pH_i assessment, the NBMD macrophages were washed twice and then resuspended in buffer A plus any other additive. At 10 min before the end of this preincubation period, the acetoxymethyl ester derivative of BCECF (2 μM) was added to the cells, which were then prepared for pH_i determinations as previously described (Whetton *et al.*, 1988b).

In some cases, ammonium prepulsing experiments (Bierman *et al.*, 1987) were performed. After preincubation and loading with BCECF, cells were treated with NH_4Cl (15 mM) for 15 min. Ammonia rapidly enters the cells and forms NH_4OH ; the extracellular ammonia is then washed away, causing the efflux of ammonia and cellular acidification as an excess of H^+ is trapped within the cells. The rapid fall in pH_i is followed by spontaneous recovery, mediated in part by the Na^+/H^+ antiport. The activity of this antiport can be measured by assessing the amiloride-sensitive rate of recovery from acute acidification (Bierman *et al.*, 1987).

RESULTS

GM-CSF but not PAF can stimulate proliferation in macrophages

NBMD macrophages have been shown to proliferate in response to GM-CSF as well as to CSF-1 and IL-3 (Whetton *et al.*, 1986; Hamilton *et al.*, 1986, 1988). We have confirmed these observations with GM-CSF as shown in Fig. 1. There is a dose-dependent increase in [3H]thymidine incorporation stimulated by GM-CSF; the ED_{50} (dose producing 50% of maximal effect) observed was 0.5 nM. The maximal rate of thymidine incorporation achieved with GM-CSF was 60% of that observed with CSF-1. PAF (0.01–1.0 μM) was unable to stimulate proliferation in NBMD macrophages.

PAF, but not GM-CSF, stimulates inositol lipid hydrolysis and Ca^{2+} mobilization

A variety of growth factors have been demonstrated to stimulate inositol lipid hydrolysis leading to proliferation; however, nothing is known about the effects of GM-CSF on this second messenger pathway. Our results show (Table 1) that although there is no change in either inositol trisphosphate or total phosphate levels following a 5 min incubation with GM-CSF, PAF-treated cells over the same time course exhibit a more than 5-fold increase in total phosphates and also a significant increase in $InsP_3$ levels. Macrophages pretreated with PAF (1 μM) for 4 h ($\pm$ GM-CSF, 1 nM) showed no increase in inositol phosphate production when washed and rechallenged with PAF, suggesting that they were desensitized to this agonist.

Stimulation of inositol lipid hydrolysis increases

Fig. 1. Effect of GM-CSF and other agents on $[^3\text{H}]$ thymidine incorporation in NBMD macrophages

Results shown are expressed as a percentage of the maximal response observed (with GM-CSF). Additives employed were: A, no addition; B, 1 pM-GM-CSF; C, 10 pM-GM-CSF; D, 0.1 nM-GM-CSF; E, 1 nM-GM-CSF; F, 1 μM -PAF; G, 0.1 μM -PAF; H, 0.01 μM -PAF; I, 1 nM-GM-CSF + 0.01 μM -5-MNIA; J, 1 nM-GM-CSF + 0.1 μM -5-MNIA; K, 1 nM-GM-CSF + 1 μM -5-MNIA; L, 1 nM-GM-CSF + 10 μM -5-MNIA; M, 1 nM-GM-CSF + 100 μM -5-MNIA. Results are the means of at least three experiments; S.D. was less than 10% in all cases.

cytosolic Ca^{2+} levels (Berridge & Irvine, 1984). As would be anticipated, PAF (1 μM) stimulated an acute increase in $[\text{Ca}^{2+}]_i$ in NBMD macrophages (Fig. 2). Other growth factors increase cytosolic Ca^{2+} levels with no concomitant increase in inositol lipid hydrolysis (Hesketh *et al.*, 1985). However, GM-CSF did not stimulate an increase in $[\text{Ca}^{2+}]_i$ in NBMD macrophages. Furthermore, pretreatment of NBMD macrophages with GM-CSF (1 nM) for between 2 min and 2 h did not alter the PAF-stimulated increase in cytosolic Ca^{2+} levels (Fig. 2).

Both PAF and GM-CSF stimulate Na^+/H^+ antiport activation

Many growth factors and other agonists stimulate an increase in pH_i via activation of an amiloride-sensitive Na^+/H^+ antiport (see Grinstein *et al.*, 1989, for review). This increase in pH_i has been shown to be a permissive signal for cellular proliferation in a variety of different cells (Johnson *et al.*, 1976), although recent evidence suggests that in renal mesangial cells, bicarbonate-linked regulation of pH_i by growth factors may be of greater relevance under physiological conditions (Ganz *et al.*, 1989; Thomas, 1989). We have investigated the regulation of this antiport in macrophages. Both PAF and GM-CSF stimulated a marked increase in pH_i within 2 min of their addition to cells in bicarbonate-containing or bicarbonate-free medium. These increases were de-

Table 1. The effect of PAF and GM-CSF on inositol phosphate levels and pH_i in NBMD

Results are means $\pm$ S.D. from at least four observations.

Addition	$[^3\text{H}]\text{InsP}_x$ after 5 min incubation (% of control)	Total $[^3\text{H}]\text{InsPs}$ after 5 min incubation (% of control)	Increase in pH_i after 20 min incubation	
			Bicarbonate-free medium	Bicarbonate-containing medium
None	(100)	(100)	0.00 $\pm$ 0.01	0.00 $\pm$ 0.01
PAF (1 μM)	164 $\pm$ 17	567 $\pm$ 9	0.08 $\pm$ 0.01	0.073 $\pm$ 0.001
GM-CSF (1 nM)	96 $\pm$ 13	96 $\pm$ 17	0.07 $\pm$ 0.02	0.07 $\pm$ 0.01

Fig. 2. Effect of PAF and GM-CSF on cytosolic Ca^{2+} levels in macrophages

GM-CSF (1 nM) or PAF (1 μM) were added to a suspension of quin-2-loaded macrophages as shown. Results are typical of at least four experiments.

Fig. 3. Effect of GM-CSF or PAF on pH_i in NBMD macrophages in (a) bicarbonate-free medium and (b) bicarbonate-containing medium

Macrophages were treated with GM-CSF (0.1 nM) or PAF (0.1 μ M) in the presence or absence of the Na^+/H^+ antiport inhibitor 5-MNIA (10 μ M). Results shown are representative of at least four experiments.

pendent on the presence of extracellular Na^+ , and were markedly inhibited by the amiloride analogue 5-(*N*-methyl-*N*-isobutyl)amiloride (5-MNIA) (Fig. 3). These results suggest that pH_i changes stimulated by both PAF and GM-CSF are mediated predominantly by the amiloride-sensitive Na^+/H^+ antiport.

The dose-effect curve for GM-CSF-stimulated pH_i increases was similar to that for GM-CSF-stimulated [3H]thymidine incorporation (Fig. 4). However, as mentioned previously, there is no effect of PAF on macrophage proliferation. One possible explanation for this is that Na^+/H^+ antiport activation has no role in mononuclear phagocytic cell proliferation. To investigate this further we have employed several distinct approaches.

First we investigated the effects of 5-MNIA on GM-CSF-stimulated DNA synthesis. There was a marked inhibition of proliferation by this agent (see Fig. 1). The ID_{50} (concn. causing 50% inhibition) for this effect was 10 μ M, very similar to that observed for the inhibition of IL-3-stimulated haemopoietic stem cell proliferation (Whetton *et al.*, 1988*b*). This supports the contention that the effect of 5-MNIA on cellular proliferation is achieved through its ability to inhibit Na^+/H^+ antiport activity and modulate pH_i . It should however be noted that the addition of sodium bicarbonate (10 mM) to the culture medium led to the abrogation of the 5-MNIA inhibition (results not shown), suggesting that an alternative form of pH_i regulation can bypass the Na^+/H^+ antiport blockade using 5-MNIA (see Grinstein *et al.*, 1989).

Secondly, the cells were incubated with either PAF or GM-CSF for 4 h and any change in resting pH_i levels as a result of these incubations was assessed. The pH_i recorded for those cells pretreated with the growth factor in the absence of bicarbonate was 0.21 ± 0.07 pH units (mean $\pm$ s.d., $n = 4$) above that obtained for cells either maintained in medium containing PAF or incubated in 10% horse serum in Hepes-buffered Fischer's medium (Table 2). Similar differences in pH_i were observed when this experiment was performed in the presence of bicarbonate (Table 2). This lack of ability of PAF to maintain a sustained increase in pH_i may be related to the desensitization of the NBMD macrophages to this agonist (see above).

Thirdly, the rate of 5-MNIA-sensitive recovery from acute acidification (Fig. 5) demonstrated a more active Na^+/H^+ antiport in those cells pretreated with GM-CSF than in cells preincubated with PAF. Furthermore, the growth-factor-treated cells returned to a higher pH_i

Fig. 4. Effect of various concentrations of GM-CSF on pH_i of NBMD macrophages

These results were obtained in bicarbonate-free medium; similar results were obtained when bicarbonate was also included (see the Materials and methods section). GM-CSF was added to macrophages at concentrations of: (a) 1 nM, (b) 0.1 nM, and (c) 0.01 nM. Results shown are typical of at least three experiments.

Table 2. Effect of PAF and GM-CSF on pH_i after 4 h of incubation

Results are expressed as the increase in pH_i compared with the pH_i of control cells incubated for 4 h in the absence of GM-CSF or PAF. Results shown are the means $\pm$ S.D. from four observations.

Addition	pH_i compared with control incubation in bicarbonate-free medium	pH_i compared with control incubation in bicarbonate-containing medium
PAF (1 μM)	$+0.01 \pm 0.01$	$+0.08 \pm 0.06$
GM-CSF (1 μM)	$+0.21 \pm 0.07$	$+0.26 \pm 0.08$

Fig. 5. Recovery of NBMD macrophages from acute acidification

Macrophages were pretreated for 4 h with GM-CSF (1 nM), PAF (1 μM) or no addition. After this time, cells were loaded with BCECF and then acid-loaded (see the Materials and methods section). The recovery from this acid loading was then monitored in the presence and the absence of 5-MNIA (10 μM). A representative trace of GM-CSF-pretreated cells recovering from acid loading in the presence of 5-MNIA is shown. Similar results were obtained in cells pretreated with PAF, or no addition. These experiments were all performed in bicarbonate-free medium. Results shown are typical traces from at least three experiments.

following acid loading than the PAF-treated or control cells (Fig. 5). GM-CSF can apparently maintain a sustained increase in the level of Na^+/H^+ antiport activity, whereas PAF has only a transient effect on this antiport.

DISCUSSION

GM-CSF can stimulate the proliferation of NBMD macrophages, but the mechanism whereby the growth factor achieves this response remains unclear. The results presented here have demonstrated that GM-CSF does not stimulate Ca^{2+} mobilization or the hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$. Some growth factors, however, potentiate inositol lipid hydrolysis which is stimulated by a secondary growth factor, and this may be associated with their mitogenic effects (Heslop *et al.*, 1986; Pouyssegur *et al.*, 1988). Preincubation with GM-CSF (1 nM, for 1 min to 2 h) was unable to modulate the response of macrophages to PAF. Clearly, inositol lipid hydrolysis has little or no role in GM-CSF-stimulated proliferation. However, it does induce an increase in pH_i via the activation of the amiloride-sensitive Na^+/H^+ antiport in the presence or absence of bicarbonate. Clearly, GM-CSF, like some other growth factors (Vara & Rozengurt, 1985), can stimulate the Na^+/H^+ exchanger without inducing inositol lipid hydrolysis. Since the observed mitogenic effect of GM-CSF is lost on treating the cells with the amiloride analogue 5-MNIA in bicarbonate-free medium, it can be implied that this cytoplasmic alkalization may be associated with the GM-CSF-stimulated mitogenic response. Our observation that this inhibition can be relieved by bicarbonate infers that other means of modulating pH_i can be employed when this antiport is inhibited. In this respect it should be noted that recent data indicate that in renal mesangial cells, growth factors may stimulate bicarbonate-dependent mechanisms to both increase and decrease pH_i (see Ganz *et al.*, 1989; Thomas, 1989). Our observations using NBMD macrophages indicate that even in the presence of bicarbonate, acute changes in pH_i in response to GM-CSF and PAF are predominantly mediated by activation of the Na^+/H^+ antiport.

Mitogen-stimulated changes in pH_i have been implicated in the control of cellular proliferation in other cell types (Johnson *et al.*, 1976; Grinstein *et al.*, 1989). Yet PAF, which is not a proliferative stimulus for macrophages, can also induce an acute increase in pH_i in these cells, probably via the activation of protein kinase C (Moolenaar, 1986). However, macrophages exposed to PAF for 4 h or more exhibit a pH_i level similar to that of untreated cells. This suggests that PAF induces only an acute, transitory increase in intracellular pH_i prior to the desensitization of the cells to this agonist (see Table 2 and the Results section), whereas long-term exposure of macrophages to GM-CSF leads to a sustained rise in pH_i and activation of the Na^+/H^+ antiport. This distinct difference in the duration of the activation of the Na^+/H^+ antiport may indicate two distinct functions for this protein in macrophages: to remove protons generated during the oxidative burst stimulated by agonists such as

PAF (Huang *et al.*, 1988) and to act to increase pH_i as a permissive signal in cellular proliferation (Moolenaar, 1986; Grinstein *et al.*, 1989). Vairo & Hamilton (1988) have also observed Na^+/H^+ antiport activation in NBMD macrophages treated with M-CSF, inferring that this may be a common event stimulated by macrophage growth factors.

Haemopoietic growth factors (GM-CSF and G-CSF) which act upon (post-mitotic) neutrophils to enhance their functional activity (Begley *et al.*, 1986; Kitagawa *et al.*, 1987) have no stimulatory effect on the Na^+/H^+ antiport (Sullivan *et al.*, 1987; Naccache *et al.*, 1988), although M-CSF, GM-CSF and G-CSF stimulate Na^+/H^+ antiport activation in the common progenitor cell for macrophages and neutrophils, GM-colony-forming cells (Cook *et al.*, 1989). Clearly the responses of macrophages, which still have proliferative potential, and of post-mitotic polymorphonuclear phagocytes to the haemopoietic growth factors may be different. Whether this differential response to GM-CSF is due to the expression of different receptor subtypes on the two types of cell awaits further characterization of the receptor(s) for this growth factor, but it is an attractive hypothesis that progenitor cells may express haemopoietic growth factor receptors which elicit intracellular events leading to proliferation, whereas post-mitotic cells express receptors which elicit signals associated with mature cell chemotaxis to sources of haemopoietic growth factors (Gasson *et al.*, 1984) and priming of mature cell function (Weisbart *et al.*, 1987; Naccache *et al.*, 1988). In this respect, the macrophage (or monocyte) differs from the other myeloid cells in that it retains proliferative potential on release from the bone marrow and within the tissues (Stanley *et al.*, 1983; Chen *et al.*, 1988*a,b*). This may explain why two stimuli (PAF and GM-CSF) with very different effects elicit a common activation of Na^+/H^+ exchange. It would be of interest to establish whether post-mitotic NBMD macrophages (prepared by treating the cells with interferon- γ and lipopolysaccharide; see Hamilton & Adams, 1987) also alter their responses to haemopoietic growth factors. Certainly the data we present suggest that some caution should be used in choosing the target cell population for studies on the elucidation of molecular mechanisms of haemopoietic growth factor-stimulated proliferation.

This work was funded by the Leukaemia Research Fund. S. J. V. is the recipient of a Science and Engineering Research Council studentship

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