Volume 256, number 1,2, 62–66 FEB 07665 October 1989

Granulocyte-macrophage colony-stimulating factor (GM-CSF) primes the respiratory burst and stimulates protein biosynthesis in human neutrophils

Steven W. Edwards, Charlotte S. Holden, John M. Humphreys and C. Anthony Hart†

Departments of Biochemistry and †*Medical Microbiology, University of Liverpool, PO Box 147, Liverpool L69 3BX, England*

Received 14 July 1989

Pre-treatment of human neutrophils with rGM-CSF resulted in a 3-fold increase in the rate of fMet-Leu-Phe stimulated reactive oxidant generation, as assessed by luminol- and lucigenin-chemiluminescence and O_2^- secretion. When bloodstream neutrophils were incubated in RPMI 1640 medium supplemented with [^{35}S]methionine, both fMet-Leu-Phe (0.1 μM) and γ-interferon (100 U/ml) stimulated a 3–4-fold increased incorporation of label into TCA-precipitable material. Similarly, rGM-CSF (50 U/ml) also stimulated protein biosynthesis in bloodstream neutrophils, and newly labelled polypeptides were separated by two-dimensional polyacrylamide gel electrophoresis. Two classes of polypeptides were visualised on these gels: the relative rate of labelling of one class changed very little upon rGM-CSF treatment whereas the relative rate of labelling of a second group increased 3–12-fold.

Neutrophil; Granulocyte-macrophage colony-stimulating factor; Protein biosynthesis

1. INTRODUCTION

Polymorphonuclear leukocytes (neutrophils) play a crucial role in the protection of the host against microbial pathogens and hence possess a wide range of cytotoxic enzymes and associated pathways in order to fulfill this defensive function. Since the early observation [1] that neutrophils isolated from the bloodstream of patients with acute bacterial infections are functionally modified, there has been a growing interest in the mechanisms by which pathogen- or host-derived components can affect the bactericidal activity of these cells. Thus, in vitro and presumably in vivo, bloodstream neutrophils can be 'primed' into a state of enhanced functional responsiveness upon exposure to some cytokines and other components which are generated during an inflammatory response [2], so that their capacity to respond to a pathogenic challenge is greatly increased. Functional changes induced by exposure to 'priming' agents include modified chemotaxis, increased oxidant generation, up-regulation of some membrane receptors, and enhanced phagocytosis and killing.

We have previously shown that 'priming' of bloodstream neutrophils with either low concentrations of the chemotactic peptide, fMet-Leu-Phe [3] or γ-interferon [4,5], results in a transition to an enhanced functional state and that this is accompanied by a 3–4-fold increase in the rate of [^{35}S]methionine incorporation into proteins. This latter phenomenon was neither due to monocyte contamination of the neutrophil suspensions nor due to changes in the intracellular pool size of [^{35}S]methionine, but was prevented by inhibitors of transcription and translation. Analysis of two-dimensional gels revealed two classes of newly labelled polypeptides, one whose rate of labelling

Correspondence address: S.W. Edwards, Department of Biochemistry, University of Liverpool, PO Box 147, Liverpool L69 3BX, England

Abbreviations: rGM-CSF, recombinant granulocyte-macrophage colony-stimulating factor; luminol, 5-amino-2,3-dihydro-1,4-phthalazinedione; fMet-Leu-Phe, *N*-formyl-methionyl-leucyl-phenylalanine

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

changed very little (1–2-fold) and another whose rate of labelling increased 10–20-fold. We have proposed that these, as yet unidentified, up-regulated proteins play a crucial role in neutrophil function during an inflammatory response.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a 22 kDa glycoprotein produced by activated T cells, endothelial cells, fibroblasts and macrophages [6–8]. It is capable of promoting increased proliferation of cells of the erythroid and myelomonocytic cell lineages, and can also induce 'priming' of mature, bloodstream neutrophils (see [9] for a review). The aim of the present work, therefore, was to determine whether in common with some other neutrophil 'priming' agents [3,5] rGM-CSF was also capable of activating de novo protein biosynthesis in circulating human neutrophils.

2. MATERIALS AND METHODS

Polymorphonuclear leukocytes (neutrophils) were isolated from heparinized venous blood from healthy volunteers utilising either a dextran/ficoll sedimentation procedure [10] or Mono-Poly Resolving Medium (Flow Laboratories) as described in the manufacturer's instructions. After purification, cells were suspended either in a Krebs/Hepes buffer containing (mM): 120 NaCl; 4.8 KCl; 1.2 KH_2PO_4; 1.3 $CaCl_2$; 1.2 $MgSO_4$; 25 Hepes (pH 7.4) and 0.1% bovine serum albumin, or RPMI 1640 medium (Flow Laboratories). Neutrophils (>98% purity) were counted using a Fuchs-Rosenthal haemocytometer slide and used within 5 h of preparation.

2.1. *Measurement of [^{35}S]methionine incorporation*

Neutrophils were suspended in RPMI 1640 medium to 2–4 × 10^7 cells/ml at 37°C. To each incubation mixture, 60 μCi/ml (final conc.) of [^{35}S]methionine was added and neutrophils were maintained in suspension by gentle agitation. After a 10 min pre-incubation period, fMet-Leu-Phe, γ-interferon or rGM-CSF were added (at the stated concentrations), whilst control suspensions contained no additions or else 0.01% DMSO (used as the solvent vehicle for fMet-Leu-Phe). After 60 min incubation, portions were removed and proteins precipitated with 10% TCA (final conc.) containing 2% (w/v) casein hydrolysate for 16 h at 4°C. Precipitated proteins were then filtered onto Whatman GF/C filters, washed six times with 10% TCA and finally once with ethanol. The filters were then dried, mixed with 4 ml of Scintillation Cocktail T (BDH Chemicals) and counted using a Packard Scintillation Counter.

2.2. *Two-dimensional polyacrylamide gel electrophoresis*

Neutrophils were suspended in RPMI 1640 medium containing 60 μCi/ml [^{35}S]methionine and incubated for 60 min at 37°C. After this period proteins were precipitated with 10% TCA for 16 h at 4°C and then centrifuged at 11600 × g for 5 min. The supernatants were discarded and the protein precipitates washed five times with 1 ml aliquots of ether (to remove traces of TCA). After the final wash and removal of supernatants, the pellets were warmed to 37°C to remove residual traces of ether. Protein precipitates were then analysed by two-dimensional gel electrophoresis employing a system utilizing isoelectric focusing (IEF) [11] for the first dimension: the second dimension employed a 13% polyacrylamide gel containing SDS. After electrophoresis, gels were soaked in DMSO for 3 h (with 3 changes) prior to soaking in a PPO (2,5-diphenyl oxazole) solution in DMSO (20%, w/w, final conc.). After extensive washing in double-distilled water, gels were dried and exposed to pre-flashed Fuji RX X-Ray film at −70°C for 2–3 weeks.

2.3. *Reactive oxidant generation*

Suspensions of neutrophils (1–2 × 10^6 cells/ml) were incubated with 10 mM luminol as described previously [12] and chemiluminescence was measured using an LKB Wallac 1250 luminometer. Rates of O_2^- production were measured as superoxide dismutase-inhibitable cytochrome c reduction [13].

3. RESULTS

3.1. *Effect of rGM-CSF and γ-interferon on oxidant generation*

Neutrophil suspensions were incubated at 37°C for 15 min in the presence or absence of γ-interferon (100 U/ml) or rGM-CSF (50 U/ml) and then stimulated by the addition of 1 μM (final conc.) fMet-Leu-Phe (suitable experiments showed that these concentrations of γ-interferon and rGM-CSF produced maximal priming of oxidant production). Fig.1 shows that γ-interferon pre-treatment resulted in a 2-fold increase in the total chemiluminescence generated after stimulation by fMet-Leu-Phe, whereas a greater enhancement (3-fold) was observed upon pre-treatment with rGM-CSF. Similar effects were observed when oxidant generation was measured using lucigenin-dependent chemiluminescence (data not shown). Similarly, γ-interferon primed fMet-Leu-Phe stimulated O_2^- secretion (fig.2), but again the priming effect induced by rGM-CSF was greater.

3.2. *Effects of priming agents on rates of protein biosynthesis*

As rGM-CSF induced a similar effect to γ-interferon on stimulated oxidant generation, we next examined its effects on protein biosynthesis. Thus, bloodstream neutrophils were incubated in RPMI 1640 medium supplemented with 60 μCi/ml [^{35}S]methionine, and after 60 min incubation at 37°C radioactivity incorporated into TCA-precipitable material was measured. Control in-

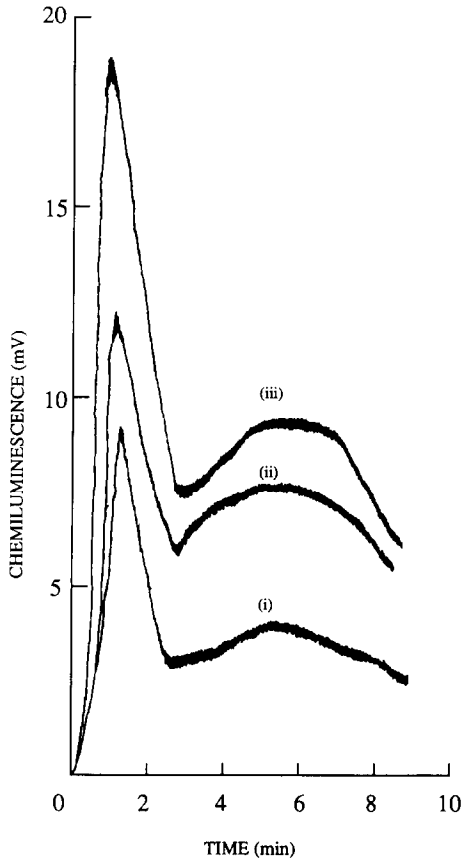

Fig.1. Effect of rGM-CSF and γ-interferon on fMet-Leu-Phe chemiluminescence. Neutrophils were suspended in Krebs/Hepes buffer at 10^6 cells/ml containing 10 μM luminol. After 15 min incubation at 37°C in the absence (i) or presence of 100 U/ml γ-interferon (ii) or 50 U/ml rGM-CSF (iii), suspensions were stimulated by the addition of 1 μM (final conc.) fMet-Leu-Phe. Similar results were obtained in at least 5 other separate experiments.

cubations contained either no additions or 0.01% DMSO (used as a solvent vehicle for fMet-Leu-Phe) whereas primed suspensions contained either rGM-CSF (50 U/ml), γ-interferon (100 U/ml) or fMet-Leu-Phe (0.1 μM). Compared to the low levels of [^{35}S]methionine incorporated into control suspensions, all of the priming agents used resulted in a marked and reproducible stimulation of incorporation (table 1).

3.3. *Two-dimensional polyacrylamide gel electrophoresis*

We have previously shown that the polypeptides synthesised after exposure of neutrophils to either

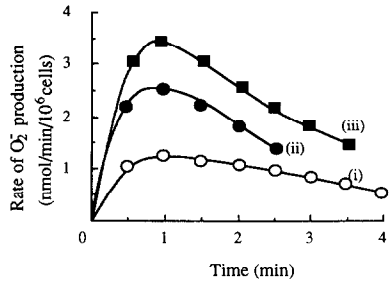

Fig.2. Effect of priming agents on O_2^- production. Neutrophils were suspended in Krebs/Hepes buffer containing 75 μM cytochrome *c* at 5×10^5 cells/ml. Other experimental conditions were exactly as described for fig.1.

fMet-Leu-Phe [3] or γ-interferon [5] fall into one of two classes, namely one group whose rate of labelling changes very little (1–2-fold) and a second whose rate of labelling increases 10–20-fold. We next examined, therefore, whether rGM-CSF induced similar changes in the polypeptide profiles of these cells. Bloodstream neutrophils were thus incubated in the presence or absence of rGM-CSF for 60 min and newly-labelled polypeptides were analysed by two-dimensional polyacrylamide gel electrophoresis. Fig.3 shows that in control suspensions a number of [^{35}S]methionine-labelled polypeptides were observed, whereas in rGM-CSF-treated suspensions a greater number of labelled polypeptides were detected and the intensity of

Table 1

Stimulation of protein biosynthesis by some neutrophil priming agents

Compound	Concentration	Increased rate of protein biosynthesis
fMet-Leu-Phe	0.1 μM	3.6 ± 1.7 (n = 9)
γ-Interferon	100 U/ml	3.2 ± 1.4 (n = 9)
rGM-CSF	50 U/ml	2.2 ± 0.5 (n = 9)

Neutrophils were suspended in RPMI 1640 medium supplemented with 60 μCi/ml [^{35}S]methionine at $2–4 \times 10^7$ cells/ml. Suspensions contained no further additions (or 0.01% DMSO as solvent vehicle for fMet-Leu-Phe) or else were treated with the neutrophil priming agents at the concentrations indicated. After 60 min incubation at 37°C proteins were precipitated and incorporated radioactivity counted, as described in section 2. Values presented are means of the fold incorporation over control suspensions (with standard deviations). n, number of determinations

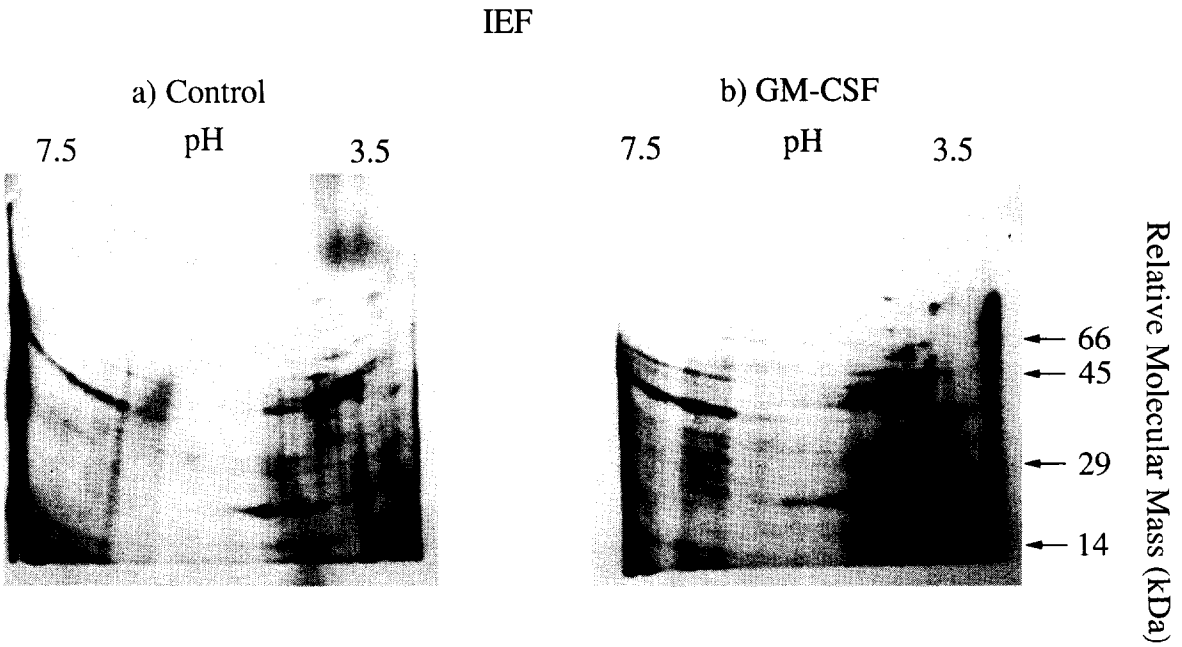

Fig.3. Two-dimensional polyacrylamide gel electrophoresis. Neutrophils were suspended at 2–4 × 10^7 cells/ml in RPMI 1640 medium containing 60 μCi/ml [^{35}S]methionine for 60 min in the absence (a) or presence (b) of 50 U/ml rGM-CSF. After this incubation period, radiolabelled polypeptides were precipitated and resolved by gel electrophoresis as described in section 2.

labelling of some was greatly increased. The relative rates of labelling of twelve polypeptides were estimated by excising corresponding spots from both control and rGM-CSF-treated gels and measuring incorporated radioactivity by scintillation counting. With six of these polypeptides the ratio of labelling (cpm incorporated into rGM-CSF treated ÷ cpm incorporated into control) ranged from 0.7 to 2.0, whereas for the other six polypeptides this ratio ranged from 3.0 to 11.0.

4. DISCUSSION

The data presented in this report clearly demonstrate that in common with some other neutrophil priming agents [3–5], rGM-CSF can both prime the respiratory burst (figs 1,2) and activate de novo protein biosynthesis (table 1, fig.3) in mature, circulating neutrophils. Interestingly, analysis of [^{35}S]methionine-labelled polypeptides on two-dimensional gels revealed two classes of proteins in rGM-CSF-treated cells. The first of these comprised polypeptides whose rate of labelling increased little (if at all) after exposure to the colony-stimulating factor, whereas the second class comprised polypeptides whose rate of labelling was markedly up-regulated during priming. It thus appears that in common with our previous observations [3,5], the priming treatment induces a selective up-regulation of gene expression.

GM-CSF is of considerable clinical interest as it has the ability to increase both the number (via its effects on myelomonocytic cell proliferation) and activity (via its priming ability) of circulating neutrophils. In vitro it has been shown to enhance stimulated reactive oxidant production [14,15] and alters both the number and affinity of some neutrophil receptors [16]. It is thus of therapeutic interest for the treatment of patients with granulocyte defects or granulocytopenias induced by disease or myelosuppressive therapy, and who inevitably are susceptible to microbial infections. Clinical trials in patients with granulocytopenias, immune deficiencies or carcinomas have shown that as well as increasing the numbers of circulating granulocytes, their functional characteristics resemble those observed during priming in vitro [17–22].

The discovery that, in common with some other priming agents [3,5], rGM-CSF stimulates de novo

protein biosynthesis in mature, circulating neutrophils adds further evidence to the now growing literature that these cells do indeed possess a significant capacity for active macromolecular biosynthesis [23]. Further work is clearly necessary to identify these up-regulated proteins and to establish their biological role during an inflammatory response. It is of considerable interest to note that the GM-CSF treatment has been shown to activate the transcription of c-*fos* [24], interleukin-1 [25] and G- and M-CSF [26]. Our data show that the expression of considerably more cellular components is up-regulated during cytokine treatment. The somewhat naive view that mature neutrophils are biosynthetically incompetent must now be revised: these cells respond to inflammatory signals to generate their own set of pro- (and perhaps anti-)inflammatory signals which can serve to augment the recruitment, infiltration and proliferation of granulocytes, and hence elaborate the immune response to infection.

Acknowledgements: We thank Mersey Regional Health Authority and the Arthritis and Rheumatism Council for financial support.

REFERENCES

[1] McCall, C.E., Bass, D.A., DeChatelet, L.R., Link, A.S., jr and Mann, M. (1979) J. Infect. Dis. 140, 277–286.
[2] Edwards, S.W. (1989) Biochem. Soc. Trans. 17, 460–462.
[3] Hughes, V., Humphreys, J.M. and Edwards, S.W. (1987) Biosci. Rep. 7, 881–889.
[4] Edwards, S.W., Say, J.E. and Hughes, V. (1988) J. Gen. Microbiol. 134, 37–42.
[5] Humphreys, J.M., Hughes, V. and Edwards, S.W. (1989) Biochem. Pharmacol. 38, 1241–1246.
[6] Chan, J.Y., Slamon, D.J., Nimer, S.D., Golde, D.W. and Gasson, J.C. (1986) Proc. Natl. Acad. Sci. USA 83, 8669–8673.
[7] Munker, R., Gasson, J.C., Ogawa, M. and Koeffler, H.P. (1986) Nature 323, 79–82.
[8] Thorens, B., Mermod, J.J. and Vassalli, P. (1987) Cell 48, 671–679.
[9] Weisbart, R.H. (1989) Ann. Intern. Med. 110, 297–303.
[10] Edwards, S.W., Say, J.E. and Hart, C.A. (1987) J. Gen. Microbiol. 133, 3591–3597.
[11] O'Farrell, P.H. (1975) J. Biol. Chem. 250, 1133–1142.
[12] Edwards, S.W. (1987) J. Clin. Lab. Immunol. 22, 35–39.
[13] Babior, B.M., Kipnes, R.S. and Curnutte, J.T. (1973) J. Clin. Invest. 52, 741–744.
[14] Weisbart, R.H., Golde, D.W., Clark, S.C., Wong, G.C. and Gasson, J.C. (1985) Nature 314, 361–363.
[15] Weisbart, R.H., Kwan, L., Golde, D.W. and Gasson, J.C. (1987) Blood 69, 18–21.
[16] Weisbart, R.H., Golde, D.W. and Gasson, J.C. (1986) J. Immunol. 137, 3584–3587.
[17] Groopman, J.E., Mitsuyasu, R.T., DeLeo, M.J., Oette, D. and Golde, D.W. (1987) N. Engl. J. Med. 317, 593–598.
[18] Vadhan-Raj, S., Keating, M., LeMaistre, A., Hittleman, W.N., McCreadie, K., Trujillo, J.M., Broxmeyer, H.E., Henney, C. and Gutterman, J.U. (1987) N. Engl. J. Med. 317, 1545–1552.
[19] Champlin, R.E., Nimer, S.D., Ireland, P., Oette, D.H. and Golde, D.W. (1989) Blood 73, 694–699.
[20] Wing, E.J., Magee, D.M., Whiteside, T.L., Kaplan, S.S. and Shadduck, R.K. (1989) Blood 73, 643–646.
[21] Kaplan, S.S., Basford, R.E., Wing, E.J. and Shadduck, R.K. (1989) Blood 73, 636–638.
[22] Lieschke, G.J., Maher, D., Cebon, J., O'Connor, M., Green, M., Sheridan, W., Boyd, A., Rallings, M., Bonnem, E., Metcalf, D., Burgess, A.W., McGrath, K., Fox, R.M. and Morstyn, G. (1989) Ann. Intern. Med. 110, 357–364.
[23] Jack, R.M. and Fearon, D.T. (1988) J. Immunol. 140, 4286–4293.
[24] Colotta, F., Wang, J.M., Polentarutti, N. and Mantovani, A. (1987) J. Exp. Med. 165, 1224–1229.
[25] Lindemann, A., Riedel, D., Oster, W., Meuer, S.C., Blohm, D., Mertelsmann, R.H. and Herrmann, F. (1988) J. Immunol. 140, 837–839.
[26] Lindemann, A., Riedel, D., Oster, W., Ziegler-Heitbrock, H.W.L., Mertlesmann, R. and Herrmann, F. (1989) J. Clin. Invest. 83, 1308–1312.