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STIMULATION OF PROTEIN SYNTHESIS IN HUMAN NEUTROPHILS BY γ-INTERFERON

JOHN M. HUMPHREYS, VALERIE HUGHES and STEVEN W. EDWARDS*
Department of Biochemistry, University of Liverpool, P.O. Box 147, Liverpool L69 3BX, U.K.

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Abstract—Treatment of human, peripheral blood neutrophils with γ-interferon both "primed" their ability to generate reactive oxidants and increased their rate of protein synthesis. This increased rate of protein synthesis was greatest 60 min after the addition of 100 U/ml γ-interferon and was not due to an increased intracellular pool of radiolabelled amino acid. Analysis of the newly-synthesized polypeptides by two-dimensional polyacrylamide gel electrophoresis (PAGE) revealed two classes of proteins which were regulated by this agent. The first of these represented proteins whose rate of labelling increased very little (1–2-fold) whereas the rate of biosynthesis of a second group of proteins increased more markedly (10–20-fold). We propose that these newly-synthesized, γ-interferon regulated proteins play an important role in the function of these cells during an acute inflammatory response.

Polymorphonuclear leukocytes (neutrophils) constitute the "first line of defence" to protect the host against microbial pathogens and possess a battery of cytotoxic enzymes and associated pathways in order to perform this crucial role. These bactericidal enzymes are present in circulating neutrophils at high concentrations and thus they may be rapidly activated during phagocytosis by processes which are not thought to be dependent upon transcription and translation [1]. Indeed, since phagocytosis can occur in the presence of inhibitors of transcription and translation, and since neutrophils possess little endoplasmic reticulum and few ribosomes [2], protein synthesis by these cells is not considered to be necessary, or even possible, during an inflammatory response.

Over recent years it has become recognized that the function of bloodstream neutrophils is modulated when they are signalled to leave the circulation and migrate into tissues during acute inflammation. This process, referred to as "priming" can be achieved *in vitro* when neutrophils are incubated with low concentrations (usually 10-fold lower than those necessary to cause stimulation *per se*) of inflammatory mediators, so that their activity is enhanced when they are challenged with a second, heterologous stimulus [3–8]. Thus, "primed" neutrophils generate more reactive oxidants and are more cytotoxic than untreated bloodstream cells [9]. The molecular events underlying "priming" are largely unknown.

Several reports have shown that some of the effects of "priming" agents can be prevented by inhibitors of protein synthesis [10, 11], implying that this enhanced neutrophil responsiveness requires *de novo* macromolecular biosynthesis. Thus, a re-examination of the biosynthetic capabilities of bloodstream neutrophils is necessary if we are to understand the molecular events which occur during acute inflammation and in inflammatory disorders. In those reports where macromolecular synthesis has been carefully examined, it has been shown that fibronectin, several heat shock proteins, the *c-fos* gene product, interleukin-1 and several other unidentified components can be synthesized by neutrophils [12–18]. We have recently shown that the biosynthesis of a group of unidentified proteins is selectively enhanced when neutrophils are "primed" with the chemotactic peptide, fMet–Leu–Phe [19]. Since γ-interferon is now recognized to be a neutrophil "priming" agent, the aim of the present study was to establish whether this lymphokine mediates its effects via enhanced macromolecular biosynthesis.

* To whom correspondence should be addressed.

MATERIALS AND METHODS

Preparation of neutrophils. Polymorphonuclear leukocytes (neutrophils) were isolated from heparinized venous blood from healthy volunteers utilising either a dextran/ficoll sedimentation procedure [20] or Mono-Poly Resolving Medium (Flow Laboratories) as described in the manufacturer's instructions. After purification, cells were suspended either in a buffer containing (mM): NaCl, 120: KCl, 4.8; KH_2PO_4, 1.2; $CaCl_2$, 1.3; $MgSO_4$, 1.2; HEPES, 25 (pH 7.4) and 0.1% bovine serum albumin, or RPMI 1640 medium (Flow Laboratories) containing 0.5% foetal calf serum. Neutrophils (> 98% purity) were counted using a Fuchs–Rosenthal haemocytometer slide and used within 4 hr of preparation.

Measurement of [^{35}S]*-methionine incorporation.* Neutrophils were suspended in RPMI 1640 medium containing 0.5% foetal calf serum to 2–4 × 10^7 cells/ml at 37°. 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 preincubation period, γ-interferon was added (at the stated concentration), whilst control suspensions contained no additions. After suitable time intervals, aliquots were removed and proteins precipitated with 10% TCA (final conc.) containing 2% (w/v) casein hydrolysate for 16 hr at 4°. 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.

Determination of intracellular pool sizes. Neutrophil suspensions (2×10^7 cells/ml) were incubated in the presence or absence of γ-interferon (as described above) in RPMI 1640 medium containing 60 μCi/ml (final conc.) of [^{35}S]-methionine. Identical suspensions were similarly incubated in the presence of 10 μg/ml cycloheximide [19]. After 60 min incubation at 37° the cells in each suspension were washed in ice-cold buffer and divided into two portions. The amount of [^{35}S]-methionine incorporated into TCA precipitable material was determined in one portion (as described above) whereas the total [^{35}S]-methionine in the whole cell (i.e. the incorporated plus the intracellular free pool of amino acid) was determined directly by scintillation counting.

One- and two-dimensional polyacrylamide gel electrophoresis. Neutrophils were suspended in RPMI 1640 medium containing 0.5% foetal calf serum plus 60 μCi/ml [^{35}S]-methionine and incubated for 1 hr at 37°. After this period proteins were precipitated with 10% TCA for 16 hr at 4° 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° to remove residual traces of ether. Protein precipitates were then analysed by either one- [21] or two-dimensional gel electrophoresis employing systems utilizing either isoelectric focusing (IEF) [22] or non-equilibrium pH gradient electrophoresis (NEPHGE) [23] for the first dimension. The second dimension employed a 13% polyacrylamide gel containing SDS. After electrophoresis, gels were soaked in DMSO for 3 hr (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 Fugi RX X-Ray film at −70° for 2–3 weeks.

Chemiluminescence measurements. Suspensions of neutrophils ($1–2 \times 10^6$ cells/ml) were incubated with 10 μM luminol (5-amino-2,3-dihydrophthalazine-1,4-dione) as described previously [24] and chemiluminescence was measured using an LKB Wallac 1250 luminometer.

Chemicals. Actinomycin D, γ-interferon, cycloheximide and luminol were from Sigma Chemical Co. (London, U.K.) whereas [^{35}S]-methionine was from Amersham International (Amersham, U.K.). All other chemicals were of the highest purity available.

RESULTS

Effect of γ-interferon on oxidant generation

When suspensions of neutrophils were incubated at 37° for 10 min and then activated by the addition of the chemotactic peptide, fMet–Leu–Phe at 1 μM, the rate of luminol-dependent chemiluminescence (due to activated oxidant generation) increased rapidly and reached a maximum value within 30 sec of addition of stimulus (Fig. 1). However, when identical suspensions were pre-incubated with 100 U/ml of γ-interferon for 10 min prior to the addition of fMet–Leu–Phe, the rate of chemiluminescence was stimulated 2.2-fold above control values. The addition of γ-interferon alone, irrespective of concentration or time of incubation did not itself stimulate chemiluminescence, confirming its role as a neutrophil "priming" agent.

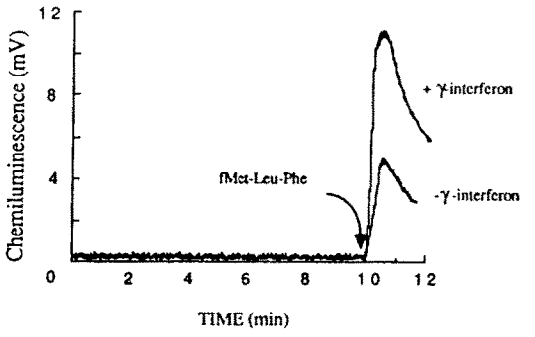

Fig. 1. Effect of γ-interferon on neutrophil chemiluminescence. Suspensions of neutrophils (10^6/ml) were incubated with 10 μM luminol in buffer at 37° in the presence or absence of 100 U/ml γ-interferon. At the time indicated the suspensions were stimulated by the addition of 1 μM fMet–Leu–Phe and the chemiluminescence responses measured.

Table 1. Time course of γ-interferon stimulated protein synthesis

Time (min)	CPM incorporated
10	2370 ± 490
30	18950 ± 7470
60	24590 ± 6480
90	13380 ± 5890

Neutrophil suspensions (2×10^7/ml, total vol. 0.5 ml) were incubated in the presence or absence of 100 U/ml γ-interferon, as described in Materials and Methods. At suitable time intervals, portions were removed (equivalent to 2×10^6 cells) from both the control (untreated) and γ-interferon treated suspensions, and after precipitation with TCA, incorporated [^{35}S]-methionine was measured by scintillation counting. Values presented have been corrected after subtraction of control counts (which were within the range 400–8500 cpm) and represent mean values plus standard deviations (N = 4).

Time course of γ-interferon stimulated protein synthesis

In order to ascertain the optimal time for stimulated protein synthesis, neutrophil suspensions were incubated in RPMI medium (containing 0.5% FCS and 60 μCi/ml [^{35}S]-methionine) in the presence or absence of 100 U/ml γ-interferon: at suitable time intervals portions were removed and the amount of labelled methionine incorporated into TCA-precipitable material was measured. Table 1 shows that the greatest rate of incorporation was observed 60 min after the addition of γ-interferon. Suitable control experiments confirmed that this increased

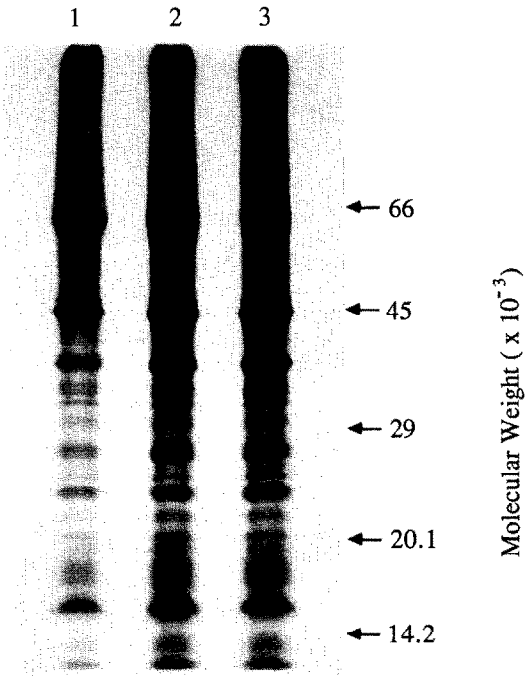

Fig. 2. PAGE of [^{35}S]-labelled polypeptides. Neutrophil suspensions (6×10^6 cells/ml, total vol. 1 ml) were incubated as described in Materials and Methods in either the absence (track 1), or presence of 0.1 μM fMet–Leu–Phe (track 2) or 100 U/ml γ-interferon (track 3). After 1 hr incubation at 37° proteins were precipitated and resolved by SDS–PAGE.

protein synthesis was not due to contaminating monocytes in the neutrophil suspensions, as described in [19]. The possibility that this enhanced rate of protein synthesis was due to increased intracellular pool sizes was investigated as described in Materials and Methods. In control and γ-interferon-treated suspensions in the presence or absence of cycloheximide, there was no significant difference in intracellular free pool levels: e.g. free pool levels (i.e. total cellular counts − incorporated counts) corresponded to 42390 ± 15490 cpm and 46310 ± 7910 cpm (N = 7) for control and γ-interferon-treated suspensions respectively.

Separation of newly-synthesized proteins by PAGE

Suspensions of neutrophils were incubated for 1 hr in the presence or absence of 100 U/ml γ-interferon or 0.1 μM fMet–Leu–Phe and then labelled proteins were precipitated and analysed by SDS–PAGE: under these conditions, this concentration of FMLP only stimulated the oxidase by 8% [19]. Figure 2 shows that in control, untreated neutrophils several polypeptides were labelled whereas in both fMet–Leu–Phe and γ-interferon "primed" cells, the overall rate of labelling was considerably greater. The major polypeptides labelled had apparent molecular weights of 60, 45, 36 and 15 kD but few, if any, differences in the polypeptide profiles were observed in the cells treated with the two "priming" agents. Also, it was not possible using this method to identify polypeptides whose expression was only detectable in "primed" cells.

Therefore, in order to resolve newly-synthesised proteins more thoroughly, 2-dimensional PAGE was employed, utilizing either IEF or NEPHGE for the first dimension to separate acidic/neutral and basic proteins, respectively. Neutrophil polypeptides labelled for 1 hr in the presence of 100 U/ml γ-interferon were compared to control suspensions incubated under identical conditions but in the absence of this lymphokine. In control neutrophils few radiolabelled polypeptide spots were visualised (Fig. 3a, c), whereas in γ-interferon "primed" suspensions considerably more polypeptides were labelled (Fig. 3b, d). The labelled polypeptides in γ-interferon "primed" cells appeared to belong to one of two classes (as has been described for fMet–Leu–Phe "primed" neutrophils, [19]) and distinguished as those whose rate of labelling either changed very little or increased markedly after "priming". The relative rate of labelling of representative polypeptides was quantified by excising labelled spots from the gels (together with suitable sections of gel devoid of labelled polypeptides to correct for background) and the ratios of counts incorporated into "primed" spots compared with control spots are shown in Table 2. This shows that the rates of labelling of some polypeptides only increased 1–2-fold, whereas others increased 10–20-fold after "priming" with γ-interferon.

DISCUSSION

The data presented here clearly show that in common with "priming" concentrations of fMet–Leu–Phe [19], γ-interferon increases the rate of protein synthesis in mature, circulating human neutrophils. The maximal stimulation of protein synthesis occurred within 1 hr of addition of this lymphokine (under the experimental conditions employed in this study) and when newly-synthesized proteins were separated by 2-dimensional PAGE two classes of proteins could be identified. These were (a) a group whose rate of labelling changed very little (i.e. 1–2-fold) and (b) a class of proteins whose rate of biosynthesis increased greatly (i.e. 10–20-fold) after treatment with γ-interferon. The data presented here further dispel the commonly-held belief that blood-stream neutrophils possess negligible capacity for macromolecular biosynthesis. Furthermore, this work shows that in addition to the previously identified components known to be actively-synthesized by these cells, the biosynthesis of a much larger group of proteins can be rapidly "up-regulated" by inflammatory mediators.

A wide variety of lymphokines, monokines, growth factors and other substances have now been shown to modulate the functional activity of mature neutrophils, although the molecular mechanisms responsible have not yet been fully investigated. Human γ-interferon has been demonstrated to evoke a number of responses in human neutrophils prior to activation by a second, heterologous stimulus. Thus, incubation with this agent enhances: antibody-dependent cytotoxicity; reactive oxidant generation; bactericidal and fungicidal activity; expression of high affinity Fc receptors; granule enzyme release [9–11, 25–29]. In addition, it has also been reported

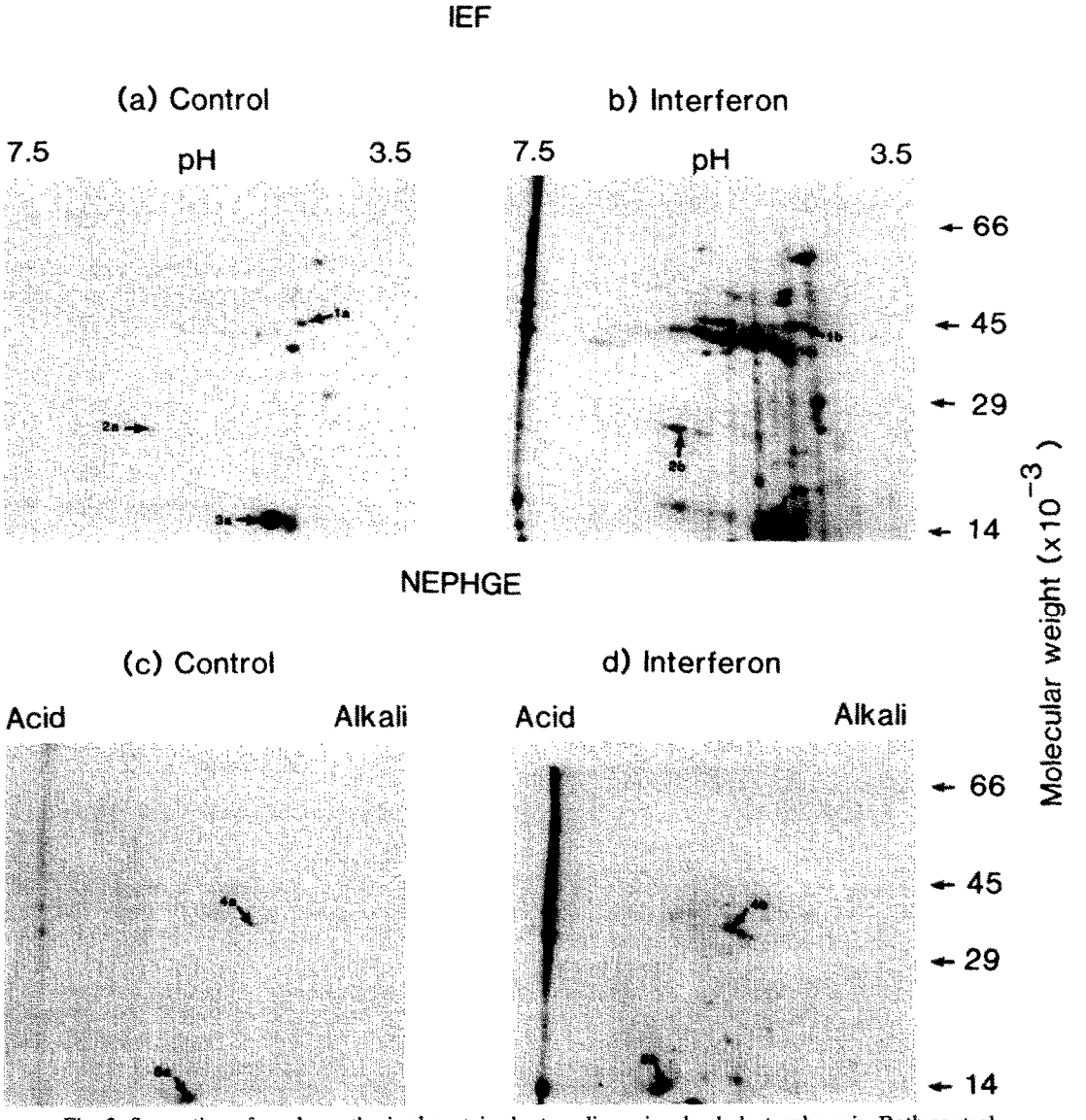

Fig. 3. Separation of newly-synthesized proteins by two-dimensional gel electrophoresis. Both control and γ-interferon treated suspensions (100 U/ml) were incubated as described in Materials and Methods. After 1 hr incubation at 37°, proteins were precipitated with TCA and then analysed by two-dimensional gel electrophoresis, utilizing either iso-electric focusing (IEF) or non-equilibrium pH gradient electrophoresis (NEPHGE) for the first dimension. The second dimension employed a 13% polyacrilamide gel (containing SDS). After electrophoresis, [^{35}S]-methionine labelled polypeptides were visualized by fluorography. Molecular weights were determined from suitable protein markers. (a, c) control, (b, d) γ-interferon treated suspensions.

that γ-interferon can also partially correct for the defect in oxidative metabolism in certain types of "variant" X-linked chronic granulomatous disease phagocytes [30]. The increased gene expression in human neutrophils is a further example of the effects that this agent can exert upon activated transcription in a variety of cell types [31–34].

The observations that (a) mature neutrophils have the capacity to synthesize macromolecules and (b) "priming" agents such as fMet–Leu–Phe and γ-interferon can enhance the rates of biosynthesis of a selective group of proteins, have far-reaching implications for our understanding of the molecular events which occur during an acute inflammatory response. Since γ-interferon also enhances reactive oxidant generation and cytotoxicity in neutrophils, we propose that these newly-synthesized proteins play a crucial role in the enhanced responsiveness of these "primed" cells during inflammation. However, it must be stressed that preincubation with γ-interferon for a minimum time period of 10 min results in maximal potentiation of the respiratory burst, whereas in this study maximal rates of protein synthesis occurred 60 min after the addition. The effect of this agent on

Table 2. Changes in rates of polypeptide labelling after treatment with γ-Interferon

Polypeptide number	Relative rate of labelling
1	2.7
2	20.2
3	2.1
4	5.5
5	2.6

Representative polypeptides from Fig. 3, were excised from the gel after fluorography, together with an equal area of gel which was devoid of labelled polypeptide to allow for background correction. Samples were mixed with 4 ml of Scintillation Cocktail T and counted for 5 min. The relative rate of labelling is expressed as:

$$\frac{\text{cpm in labelled spot b—background}}{\text{cpm in labelled spot a—background}}.$$

increased microbial killing was greatest, however, after 45–60 min [9]. Therefore, it seems likely that there are both short-term (e.g. 10 min or greater) and longer-term (up to 1 hr) priming effects on neutrophil responsiveness mediated by this agent. Further work is clearly necessary to identify these γ-interferon regulated proteins and to establish their biological function.

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