Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 2503–2507, April 1985
Medical Sciences

Selective enhancement of the expression of granulocyte functional antigens 1 and 2 on human neutrophils

(surface antigens/*N*-formyl-Met-Leu-Phe/lipopolysaccharide/β_2-microglobulin)

M. A. VADAS, A. F. LÓPEZ, AND D. J. WILLIAMSON

Experimental Allergy Laboratory of the Clinical Research Unit, The Walter and Eliza Hall Institute of Medical Research, Post Office, Royal Melbourne Hospital, Victoria 3050, Australia

Communicated by J. F. A. P. Miller, December 7, 1984

ABSTRACT **The regulation of expression of two human granulocyte functional antigens (GFA-1 and GFA-2) was examined. *N*-formylmethionylleucylphenylalanine (fMet-Leu-Phe) caused a rapid, dose-dependent enhancement of the expression of these antigens, 2- to 4-fold within 30 min, but not of another surface structure, β_2-microglobulin. Pretreatment of the cells with cytochalasin B at 5 μg/ml further enhanced the effect of fMet-Leu-Phe on the expression of GFA-2, raising its surface expression 11-fold. Lipopolysaccharide also stimulated the expression of GFA-1 and GFA-2. The effect of lipopolysaccharide was less than that of fMet-Leu-Phe and was more marked on GFA-1 than on GFA-2. Pretreatment of neutrophils with fMet-Leu-Phe not only stimulated their cytotoxic activity against antibody-coated target cells but also increased their capacity to be stimulated by monoclonal antibodies to GFA-1 and GFA-2. These findings show that the expression of functional surface structures on human neutrophils is subject to rapid and selective regulation.**

We have described recently two granulocyte-specific antigens that appear to be involved in the function of human neutrophils and eosinophils. The first of these granulocyte functional antigens (GFA-1) has a molecular weight of 110,000, and an IgM monoclonal antibody (MAb) against it strongly enhanced antibody-dependent cytotoxicity of tumor cells by human neutrophils and eosinophils (1). GFA-1 appears early in granulocyte maturation, being present on day 7 colony-forming cells (unpublished results) and seems to possess a similar reactivity to that of other anti-granulocyte IgM MAbs (2–4).

The second GFA, GFA-2, has a molecular weight of 95,000, with ≈20,000 cell-surface structures on each neutrophil. An IgG1 MAb against GFA-2 inhibited antibody-dependent cell-mediated cytotoxicity (ADCC) by neutrophils as well as eosinophils; however, the $F(ab')_2$ fragment of this MAb was a strong stimulator of these functions (5). Significantly, the capacity of neutrophils to phagocytose IgG-coated particles also was inhibited by the whole IgG but stimulated by its $F(ab')_2$ fragment.

The function of human neutrophils is also influenced by other stimuli, such as chemotactic peptides (6), colony-stimulating factors (CSF) (7), and lipopolysaccharide (LPS) (8). The effects of these substances on granulocytes include the enhancement of their tumoricidal and microbicidal capacities (9, 10), stimulation of oxidative metabolism (9, 11), and alteration of their ability to adhere to endothelial cells (12).

The mechanisms of action of these regulators are not clear, but our demonstration of the functional importance of GFA-1 and GFA-2 raised the possibility that such regulators may exert their effects by modulating these antigens. In this communication we show that the number of cell-surface GFA could be increased rapidly and selectively by the chemotactic peptide *N*-formylmethionylleucylphenylalanine (fMet-Leu-Phe) and that this was accompanied by increased stimulation of neutrophil function by MAb against these antigens. Treatment of neutrophils with cytochalasin B and fMet-Leu-Phe, a "complete secretagogue" (13), further enhanced the expression of GFA-2. LPS also increased their expression, but to a lesser extent than did fMet-Leu-Phe, and with relatively more effect on GFA-1.

MATERIALS AND METHODS

Medium. Eagle's minimum essential medium (GIBCO) containing 10% fetal calf serum (Flow), and antibiotics (benzylpenicillin at 60 mg/liter and streptomycin at 100 mg/liter; Glaxo, Boronia, Victoria, Aus.) was used for the separation of peripheral blood leukocytes, cytotoxicity, and immunofluorescence assays.

Purification of Human Neutrophils. Peripheral blood from normal volunteers was sedimented on dextran, followed by centrifugation on a hypertonic metrizamide (Nyegaard, Oslo) gradient as described (14). The purity of the neutrophil preparations was always >96% with eosinophils as the only contaminant.

Monoclonal Antibodies. The IgM MAb WEM-G1 (against GFA-1), and the IgG1 MAb WEM-G11 (against GFA-2) were developed as described (1, 5). The murine IgG2b MAb to β_2-microglobulin (β_2 m) was a gift from I. F. C. McKenzie (University of Melbourne). Nonbinding control murine MAb were K7 (IgM, anti-trinitrophenyl) and PB10 (IgG1, anti-chicken θ antigen), gifts from M. Kennedy (National Institute of Medical Research, London) and P. Bartlett (Walter and Eliza Hall Institute, Melbourne, Australia), respectively. Ascites containing MAb was precipitated with 40% ammonium sulfate before conjugation with fluorescein isothiocyanate (FITC) as described (15). In some experiments WEM-G11 was purified, and $F(ab')_2$ fragments were made (16) before conjugation with FITC.

Reagents. *Escherichia coli* LPS (026:B6, Difco) and stock solutions of fMet-Leu-Phe (Sigma; 1 mM in ethanol) and cytochalasin B (Sigma; at 0.5 mg/ml of dimethyl sulfoxide) were diluted in phosphate-buffered saline prior to use. The human granulocyte–macrophage colony stimulating factor α (CSF-α) was a gift from N. Nicola, prepared as described (17, 18) and used at concentrations found to be supramaximal in the colony-forming assay.

ADCC Assay. P815 tumor target cells (DBA/2, mastocytoma) were labeled with ^{51}Cr and coupled with trinitrophenyl as described (7). P815 cells (4×10^3) were then placed into

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: GFA, granulocyte functional antigen; fMet-Leu-Phe, *N*-formylmethionylleucylphenylalanine; β_2m, β_2-microglobulin; LPS, lipopolysaccharide; MAb, monoclonal antibody; ADCC, antibody-dependent cell-mediated cytotoxicity; CSF, colony-stimulating factor; FITC, fluorescein isothiocyanate.

96-well Linbro microtiter plates (Flow) containing rabbit anti-dinitrophenyl IgG (Miles-Yeda, Rehovot, Israel), neutrophils (1.2×10^5), and MAb or CSF dilutions in a total volume of 160 μl. Anti-dinitrophenyl crossreacts strongly with the trinitrophenyl determinant on target cells. After incubation for 2.5 hr at 37°C, 80 μl of supernatant was removed from each well for counting in a gamma counter. The percent cytotoxicity was determined according to the formula:

$$\text{Cytotoxicity} = \frac{\text{Experimental cpm} - \text{spontaneous release cpm}}{\text{maximum cpm} - \text{spontaneous release cpm}} \times 100,$$

where "spontaneous release" was obtained by incubating target cells in medium alone, and "maximum release" was obtained by incubating them with 5% Triton X-100.

Direct Immunofluorescence Assay. Granulocytes that had been treated in different ways were mixed at 10^7 cells per ml with FITC-MAb in microtiter plates for 45 min at 4°C. The cells were washed three times in medium and fixed (1% formaldehyde/5 mM sodium azide/2% glucose in phosphate-buffered saline) before analysis by flow cytometry.

Flow Cytometry. Fixed granulocytes were analyzed in a fluorescence-activated cell sorter (FACS II, Becton Dickinson), which was equipped with 3-decade logarithmic amplifiers in the fluorescence detection channel and linked to a PDP11 computer data-handling system. In each sample a minimum of 10,000 cells were analyzed. The fluorescence profiles obtained with most cell preparations were unimodal. In a few individuals staining with WEM-G1 yielded a bimodal distribution. After computer transformation to a linear scale, the mean and median fluorescence of each sample were calculated. The mean was defined as the sum of the fluorescence intensities of individual cells divided by the number of cells analyzed, and it is expressed in arbitrary units. The median is the fluorescence intensity below which 50% of the cells are found. Usually the mean and median were very similar (Table 1), and further results are expressed as mean fluorescence values of single determinations. This was necessary in order to handle several groups of cells treated in parallel under different conditions. In three experiments designed to test the variability between different samples treated in the same way, the standard error was <5% of the mean. Unless otherwise stated, variation is expressed as the SEM.

RESULTS

Enhancement of GFA-1 and GFA-2 Expression on Human Neutrophils by fMet-Leu-Phe. Purified human neutrophils were incubated for 30 min at 37°C with various doses of fMet-Leu-Phe, then washed at 4°C, and mixed with FITC-MAb WEM-G1 or WEM-G11 or with anti-β_2m before analysis by flow cytometry. fMet-Leu-Phe increased the expression of GFA-1 and GFA-2 in a dose-dependent fashion (Fig. 1). Ethanol in the concentrations present in the fMet-Leu-Phe preparations had no effect on the fluorescence. Incubation with fMet-Leu-Phe (0.1 μM) at 4°C was also ineffective. In contrast to the changes in GFA-1 and GFA-2 induced by fMet-Leu-Phe at 37°C, the amount of β_2m did not alter significantly during the experiment. These data are shown in Table 1, together with relevant controls.

Table 1. Selective enhancement of GFA-1 and GFA-2 expression on human neutrophils by fMet-Leu-Phe

Exp.	Fluorescent probe	Fluorescence intensity*	
		No pretreatment	0.1 μM fMet-Leu-Phe
A	Nil	4/4	4/4
	PB10 (IgG1)	5/5	5/5
	WEM-G11 (IgG1)†	15/14	25/25
	anti-β_2m (IgG2b)	39/34	38/32
B	Nil	4/4	4/4
	K7 (IgM)	8/5	8/5
	WEM-G1 (IgM)	570/464	847/754
	anti-β_2m (IgG2b)	54/49	58/55

*Mean/median in arbitrary units are shown.
†Identical results were obtained in other experiments with F(ab')$_2$ fractions of this MAb.

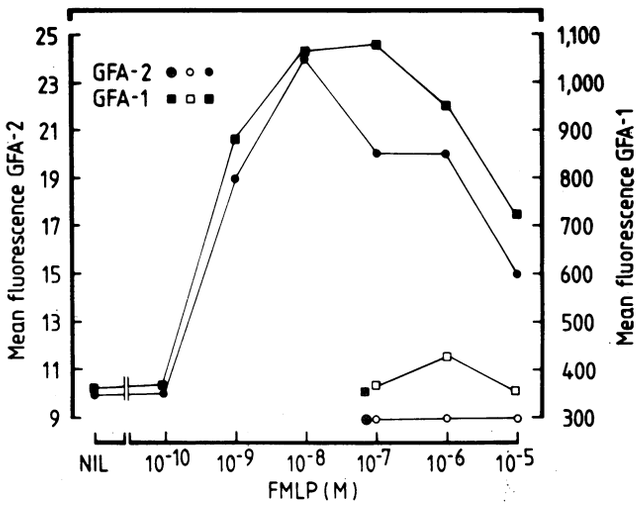

FIG. 1. Effect of incubation with various concentrations of fMet-Leu-Phe (FMLP) for 30 min at 37°C on the expression (mean fluorescence of bound FITC-MAb) of GFA-2 and GFA-1. The effect of 0.1 μM fMet-Leu-Phe for 30 min at 4°C is also shown (starred symbols), as is the effect of incubation with concentrations of ethanol found in fMet-Leu-Phe (30 min at 37°C) (open symbols).

Time Course of Enhancement of GFA-1 and GFA-2 Expression by fMet-Leu-Phe. Purified human neutrophils were incubated for various times at 37°C with or without 0.1 μM fMet-Leu-Phe, washed, and, after an aliquot was counted for viability, stained with MAb against GFA-1, GFA-2, or β_2m. As shown in Fig. 2, although there was no significant change in the expression of any antigen without fMet-Leu-Phe or of β_2m in the presence of fMet-Leu-Phe, the expression of GFA-1 and GFA-2 did increase in a time-dependent manner peaking at 30 min. The viability of the cells remained >98% throughout.

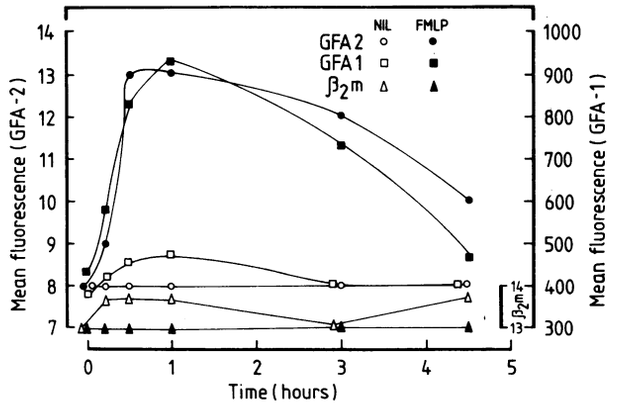

FIG. 2. Effect of incubation for various times at 37°C with or without 0.1 μM fMet-Leu-Phe (FMLP) on the expression (mean fluorescence of bound FITC-MAb) of GFA-2, GFA-1, and β_2m.

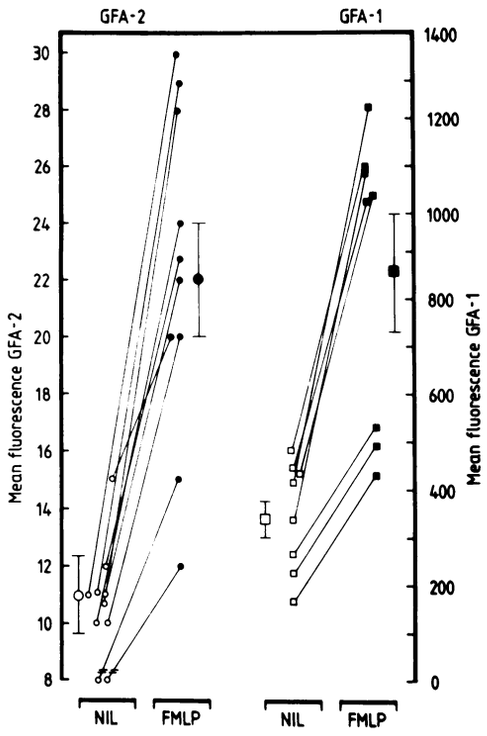

FIG. 3. Effect of incubation for 30 min at 37°C with (closed symbols) or without (open symbols) 0.1 μM fMet-Leu-Phe (FMLP) on the expression (mean fluorescence of bound FITC-MAb) of GFA-1 and GFA-2. The arithmetic mean of these values is also shown, and vertical bars span 2 SEM.

Effect of fMet-Leu-Phe on Neutrophil Surface Antigens of Different Individuals. In consecutive experiments neutrophils from 10 normal individuals were incubated with 0.1 μM fMet-Leu-Phe for 30 min at 37°C. In every case there was an increase in GFA-1 and GFA-2 expression (Fig. 3). Binding to β_2m remained essentially unchanged, the mean fluorescence being 22.3 ± 3.9 and 23.8 ± 3.6 with and without fMet-Leu-Phe, respectively.

Effect of Cytochalasin B on fMet-Leu-Phe-Mediated Enhancement of GFA Expression. In order to test whether the enhancement of GFA-1 and GFA-2 expression could be associated with degranulation, neutrophils were preincubated with cytochalasin B for 15 min at 37°C before the addition of fMet-Leu-Phe. This treatment results in rapid degranulation of primary and secondary granules. Although cytochalasin B by itself had no effect on the expression of these antigens, it enhanced the effect of fMet-Leu-Phe on GFA-2 (Fig. 4). Binding to β_2m was not significantly affected.

In five experiments, 0.1 μM fMet-Leu-Phe increased the expression of GFA-2 2- to 3-fold in the absence of cytochalasin B and about 11-fold in its presence. In contrast to the effect on GFA-2, cytochalasin B at 5 μg/ml did not enhance the expression of GFA-1 (data not shown).

Effect of LPS on the Expression of Neutrophil Surface Antigens. Incubation of neutrophils with LPS for 30 min at 37°C enhanced the expression of GFA-1 and GFA-2 but not β_2m. The enhancement was dose-dependent, but the dose that gave maximal expression varied between 10 and 100 μg/ml in different individuals. Cytochalasin B pretreatment did not enhance LPS-mediated effects (data not shown). In comparison to 0.1 μM fMet-Leu-Phe, the effect of LPS was smaller (Table 2) and was more apparent on the expression of GFA-1 than GFA-2. On average the enhancement of GFA-1 and GFA-2 expression by LPS was respectively 64 ± 14% and 32 ± 7% of that seen with fMet-Leu-Phe.

Effect of fMet-Leu-Phe on the Capacity of WEM-G1 and WEM-G11 to Stimulate Neutrophil Function. Neutrophils were incubated with or without 0.1 μM fMet-Leu-Phe for 30 min at 37°C, washed, and tested in an ADCC assay in the presence or absence of CSF-α or WEM-G1 or WEM-G11 F(ab')$_2$. In the absence of anti-target cell antibody, no significant killing took place. fMet-Leu-Phe preincubation enhanced killing by 8% in the presence of anti-target cell antibody. CSF-α caused a dose-dependent enhancement of the killing that was not significantly different whether the cells were preincubated with fMet-Leu-Phe or medium. WEM-G1

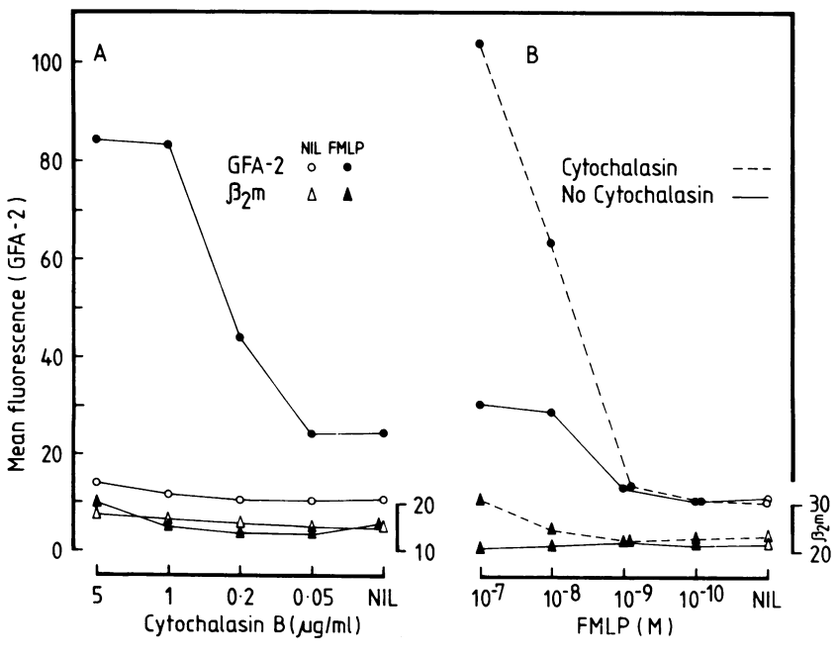

FIG. 4. (*A*) Effect of pretreatment for 15 min at 37°C with various concentrations of cytochalasin B on the expression of GFA-2 and β_2m after the addition of medium or 0.1 μM fMet-Leu-Phe (FMLP) for 30 min at 37°C. (*B*) Effect of incubation for 30 min at 37°C with various concentrations of fMet-Leu-Phe in the presence or absence of 5 μg of cytochalasin B per ml.

Table 2. Effect of LPS on the expression of GFA-1 and GFA-2 on human neutrophils

	Mean of fluorescence intensity in arbitrary units								
	No pretreatment			0.1 μM fMet-Leu-Phe			LPS at 10 or 100 μg/ml*		
Exp.	WEM-G1	WEM-G11	$\alpha\beta_2$m	WEM-G1	WEM-G11	$\alpha\beta_2$m	WEM-G1	WEM-G11	$\alpha\beta_2$m
A	215	7	14	465	13	13	273	9	13
B	264	11	13	525	22	10	430	18	10
C	413	8	10	651	25	8	649	11	10
D	335	18	37	397	34	42	407	22	39
E	415	10	29	1227	24	23	718	15	27
F	362	10	12	1085	24	10	710	13	13

*The highest values, expressed below, varied between these two doses among different individuals.

and WEM-G11 F(ab')$_2$ also caused a dose-dependent enhancement of killing but, in contrast to CSF-α, this was greater when the cells were pretreated with fMet-Leu-Phe (Fig. 5).

DISCUSSION

The central finding in this paper is that fMet-Leu-Phe, a chemotactic tripeptide derived from certain bacteria (19), caused a rapid, selective, and dose-dependent enhancement of the expression of two human neutrophil antigens, GFA-1 and GFA-2 (Figs. 1–3). These antigens have been shown to be important in some aspects of granulocyte function since MAb directed against them, WEM-G1 IgM and WEM-G11 F(ab')$_2$, respectively, strongly enhance antibody-dependent extracellular killing (1, 5) and phagocytosis (5). In contrast, the expression of β_2m was not greatly altered by fMet-Leu-Phe, although a slight increase was seen in some individuals. Control experiments failed to demonstrate an effect of fMet-Leu-Phe on neutrophil autofluorescence or the binding of MAb having the same isotype but without anti-granulocyte specificity (Table 1).

The possibility that the stimulation of GFA-1 and GFA-2 expression was related to granule movements was examined by incubating neutrophils with cytochalasin B before exposure to fMet-Leu-Phe. This regimen serves as a "complete secretagogue," causing the release of both primary and secondary granule contents (13). fMet-Leu-Phe increased the expression of both antigens 2- to 3-fold in the 10 normal subjects tested, and cytochalasin B pretreatment (5 μg/ml) caused a further 3- to 4-fold enhancement of GFA-2 expression (Fig. 4). Again β_2m expression did not change, and the effect on GFA-1 was minimal and seen only at lower concentrations (0.2 μg/ml). The suggestion from these data that GFA-1 and GFA-2 may be modulated independently was supported by the experiments using another bacterial product, LPS, as the stimulus. In this case the effect was more marked on GFA-1 than GFA-2 (Table 2) with no further increase afforded by cytochalasin B pretreatment.

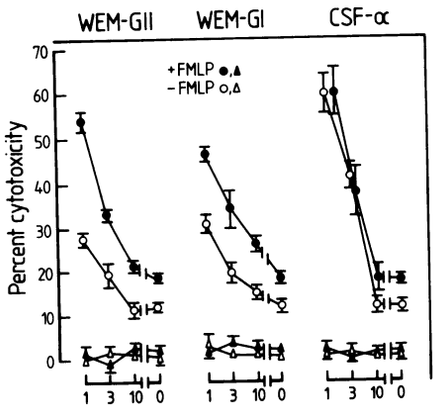

FIG. 5. Effect of pretreatment with 0.1 μM fMet-Leu-Phe (FMLP) (●, ▲) or medium (○, △) on the capacity of WEM-G11 F(ab')$_2$, WEM-G1, and CSF-α to stimulate ADCC in the presence (●, ○) or absence (▲, △) of rabbit anti-dinitrophenyl IgG. Each point is the arithmetic mean of duplicate determinations, and bars span 2 SEM. A titration with each reagent is shown, with each point expressed as the reciprocal of the dilution used. The starting concentrations were 0.5 mg/ml for WEM-G11 F(ab')$_2$, 1:10 ascitic fluid for WEM-G1, and 1:20 for CSF-α (20-fold maximal in the colony-forming assay).

The functional significance of these fMet-Leu-Phe-induced changes in GFA expression was apparent in experiments showing an increased ability of WEM-G1 and WEM-G11 F(ab')$_2$ to stimulate extracellular killing after preincubation of the neutrophil effector cells with fMet-Leu-Phe (Fig. 5). On the other hand, the stimulatory capacity of CSF-α was not enhanced. This suggests that either the mechanism whereby this latter stimulus affects extracellular killing is different from GFA-mediated effects or the putative granulocyte–macrophage CSF receptor is modulated differently.

The rapidity of the stimulated expression, the effect of cytochalasin B, and preliminary immunofluorescence studies showing intracellular staining with WEM-G11 suggest the existence of a mobile internal pool. Such a mechanism has recently been shown for the endosomal transferrin receptor of a murine macrophage cell line (20) and the secondary granule-associated cytochrome *b* of human neutrophils (21), which translocate to the cell surface in response to phorbol esters and calcium ionophores. Degranulating or chemotactic stimuli rapidly increase surface complement component C3b (22), C$_3$bi (23), and fMet-Leu-Phe (24) receptors for which intracellular pools have also been demonstrated (23, 25). Thus, it appears that there is a group of functional surface structures on phagocytes whose expression can be rapidly modulated by activating stimuli.

The function of GFA-1 and GFA-2 *in vivo* remains to be explored. A possible role in bacterial infection presents itself, either indirectly through augmentation of phagocytosis and killing or more directly as pathogen-binding sites (26). The capacity of fMet-Leu-Phe to enhance neutrophil adherence to vascular endothelium (12) and to cause sudden but transient neutropenia after intravenous injection (27) suggests an influence on margination. Recent reports of clinically significant abnormalities of leukocyte function associated with surface antigen deficiencies (28–32) and autoantibodies (33) should stimulate further efforts in this field.

We thank Ms. Lucia Callegaro and Ms. Dora Vasiliadis for excellent technical assistance and Dr. I. R. Mackay for criticizing the manuscript. This work was supported by grants from the National Health and Medical Research Council of Australia.

1. López, A. F. & Vadas, M. A. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 1818–1821.

2. Huang, L. C., Civin, C. I., Magnani, J. L., Shaper, J. H. & Ginsburg, V. (1983) *Blood* **61,** 1020–1023.
3. Skubitz, K. M., Pessano, S., Bottero, L., Ferraro, D., Rovera, G. & August, J. T. (1983) *J. Immunol.* **131,** 1882–1888.
4. Urdal, D. L., Brentnall, T. A., Bernstein, I. D. & Hakomori, S.-I. (1983) *Blood* **62,** 1022–1026.
5. López, A. F., Begley, G., Andrews, P., Butterworth, A. E. & Vadas, M. A. (1985) *J. Immunol.*, in press.
6. Snyderman, R. & Goetzl, E. J. (1981) *Science* **213,** 830–837.
7. Vadas, M. A., Nicola, N. A. & Metcalf, D. (1983) *J. Immunol.* **130,** 795–799.
8. Bradley, S. G. (1979) *Annu. Rev. Microbiol.* **33,** 67–94.
9. Issekutz, A. C., Lee, K.-Y. & Biggar, W. D. (1979) *Infect. Immun.* **24,** 295–301.
10. Vadas, M. A., Nicola, N., López, A. F., Metcalf, D., Johnson, G. & Pereira, A. (1984) *J. Immunol.* **133,** 202–207.
11. Lehmeyer, J. E., Snyderman, R. & Johnston, R. B. (1979) *Blood* **54,** 35–45.
12. Hoover, R. L., Folger, R., Haering, W. A., Ware, B. R. & Karnovsky, M. J. (1980) *J. Cell Sci.* **45,** 73–86.
13. Bentwood, B. J. & Henson, P. M. (1980) *J. Immunol.* **124,** 855–862.
14. Vadas, M. A., David, J. R., Butterworth, A., Pisani, N. T. & Siongok, T. A. (1979) *J. Immunol.* **122,** 1228–1236.
15. Goding, J. W. (1976) *J. Immunol. Methods* **13,** 215–226.
16. Parham, P. (1984) *J. Immunol.* **131,** 2895–2902.
17. Nicola, N. A., Metcalf, D., Johnson, G. R. & Burgess, A. W. (1978) *Leuk. Res.* **2,** 313–322.
18. Nicola, N. A., Metcalf, D., Johnson, G. & Burgess, A. W. (1979) *Blood* **54,** 614–627.
19. Marasco, W. A., Phan, S. H., Krutzsch, H., Showell, H. J., Feltner, D. E., Nairn, R., Becker, E. L. & Ward, P. A. (1984) *J. Biol. Chem.* **259,** 5430–5439.
20. Buys, S. S., Keogh, E. A. & Kaplan, J. (1984) *Cell* **38,** 569–576.
21. Borregaard, N., Heiple, J. M., Simons, E. R. & Clark, R. A. (1983) *J. Cell Biol.* **97,** 52–61.
22. Fearon, D. T. & Collins, L. A. (1983) *J. Immunol.* **130,** 370–375.
23. Arnaout, M. A., Todd, R. F., III, & Dana, N. (1984) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **43,** 1665 (abstr.).
24. Fletcher, M. P. & Gallin, J. I. (1980) *J. Immunol.* **124,** 1585–1588.
25. Fletcher, M. P. & Gallin, J. I. (1983) *Blood* **62,** 792–799.
26. Sharon, N. (1984) *Immunol. Today* **5,** 143–147.
27. O'Flaherty, J. T., Showell, H. J. & Ward, P. A. (1977) *J. Immunol.* **118,** 1586–1589.
28. Crowley, C. A., Curnutte, J. T., Rosin, R. E., André-Schwartz, J., Gallin, J. I., Klempner, M., Snyderman, R., Southwick, F. S., Stossel, T. P. & Babior, B. M. (1980) *N. Engl. J. Med.* **302,** 1163–1168.
29. Buchanan, M. R., Crowley, C. A., Rosin, R. E., Gimbrone, M. A. & Babior, B. M. (1982) *Blood* **60,** 160–165.
30. Arnaout, M. A., Pitt, J., Cohen, H. J., Melamed, J., Rosen, F. S. & Colten, H. R. (1982) *N. Engl. J. Med.* **306,** 693–699.
31. Dana, N., Todd, R. F., III, Pitt, J., Springer, T. A. & Arnaout, M. A. (1984) *J. Clin. Invest.* **73,** 153–159.
32. Anderson, D. C., Schmalstieg, F. C., Arnaout, M. A., Kohl, S., Tosi, M. F., Dana, N., Buffone, G. J., Hughes, B. J., Brinkley, B. R., Dickey, W. D., Abramson, J. S., Springer, T., Boxer, L. A., Hollers, J. M. & Smith, C. W. (1984) *J. Clin. Invest.* **74,** 536–551.
33. Kramer, N., Perez, H. D. & Goldstein, I. M. (1980) *N. Engl. J. Med.* **303,** 1253–1258.