1991

# Integrin Modulating Factor 1 (IMF-1): A Lipid That Modulated Leukocyte Integrins

Anne Hermanowski-Vosatka


# Integrin Modulating Factor 1 (IMF-1): A lipid that modulates leukocyte integrins

A thesis presented to the faculty of the Rockefeller University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

by

Anne Hermanowski-Vosatka

October 11, 1991
The Rockefeller University
New York, New York

# *Acknowledgements*

This thesis would not have been possible without the contributions and support of a number of people. I would like to extend my thanks....

to Sam Wright, for his unflagging optimism and enthusiasm, shrewd scientific judgement, patience and knack for saying the right thing. He has been a wonderful advisor and a great role model.

to Bob Vosatka, for his patience, understanding, helpful advice and faith in my abilities. It is a joy and a privilege to have him as a permanent collaborator.

to my parents, for their bottomless emotional support and encouragement and for their countless other gifts to me, too numerous to mention.

to my brother and sister-in-law, and to the rest of my family, especially to Stryjcio Andrzej, to whom this means so much.

to Bill Swiggard and Jos van Strijp, who were always willing to help and lend encouragement.

to Pat Detmers, and to all the past and present members of Pat's and Sam's labs, for all the things that make it a pleasure to go to work each day.

to Zanvil Cohn and Ralph Steinman for great teaching and advice.

to those who contributed technical help or reagents: to Hiroo Yuasa and Anant Menon, who helped with the purification and chemical characterization of IMF-1; to Steven Cohen, who provided the equipment for the ozonolysis; to Roy Silverstein, who collaborated on the platelet studies; to Robert Ramos and Suining Lee, who provided technical assistance with the LPS and endothelial cell assays; to a number of people in the Cohn lab, who provided buffy coat pellets, as a source of PMN; to Jack Schmidt, Jerry Liesch, Debbie Zink, Mike Goetz and Shiu Singh, who helped to obtain the GC/MS data; to Judy Adams, who generated many of the figures; to Bill Swiggard, who helped perform the chromatography; and to Jos van Strijp, who did the experiments with the isolated CR3.

# *Table of contents*

# *List of figures*

# *List of tables*

## *Abbreviations*

| | |
|---|---|
| C3bi | cleavage product of the third component of complement |
| CR3 | complement receptor type 3 |
| EC | endothelial cells |
| EC3bi | erythrocytes coated with C3bi |
| ELAM-1 | endothelial leukocyte adhesion molecule 1 |
| fMLP | formyl-methionyl-leucyl-phenylalanine |
| fNLLP | formyl-norleucyl-leucyl-phenylalanine |
| GC/MS | gas chromatography / mass spectroscopy |
| ICAM-1 | intercellular adhesion molecule 1 |
| IMF-1 | integrin modulating factor 1 |
| LAD | leukocyte adhesion deficiency |
| LAM-1 | leukocyte adhesion molecule 1 |
| LFA-1 | lymphocyte function associated antigen 1 |
| LPS | lipopolysaccharide |
| $LTB_4$ | leukotriene $B_4$ |
| $LXA_4$ | lipoxin $A_4$ |
| mAb | monoclonal antibody |
| NAP-1/IL-8 | neutrophil activating protein 1/ interleukin 8 |
| NCAM | neural cell adhesion molecule |
| PAF | platelet activating factor |
| PBS | phosphate buffered saline |
| PMA | phrobol myristate acetate |

| | |
|---|---|
| PMN | polymorphonuclear leukocytes, or neutrophils |
| SEM | standard error of the mean |
| SDS-PAGE | sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| TNF | tumor necrosis factor |
| VLA | very late activation antigen |

## *Abstract*

Polymorphonuclear leukocytes (PMN) express a receptor of the integrin family termed complement receptor type 3 (CR3, also known as $\alpha_M\beta_2$, Mac-1, Mo1 or CD11b/CD18) that functions in several cell-cell and cell-substratum adhesion events. The capacity of CR3 to mediate adhesion may be rapidly and reversibly enhanced without changes in the number of receptors expressed on the cell surface. This thesis describes an acidic, amphiphilic lipid, termed integrin modulating factor (IMF-1), that may serve to control CR3 avidity. Addition of IMF-1 to cells or to purified CR3 causes enhanced binding of ligand. IMF-1 cannot be extracted from resting PMN but can be extracted from cells within one minute of stimulation with a variety of agonists (PMA, TNF, formylated peptides, platelet activating factor). The amount of IMF-1 extracted declines to low levels within an hour of continued stimulation. The time course of IMF-1 extraction corresponds precisely with the transient increase in the adhesive activity of CR3 observed in PMN stimulated with these agonists. IMF-1 can also increase the binding activity of another leukocyte integrin, LFA-1 ($\alpha_L\beta_2$, CD11a/CD18). However, IMF-1 does not affect the function of representative $\beta_1$ and $\beta_3$ integrins. IMF-1 has a molecular weight of 340 $\pm$ 16 daltons by size exclusion chromatography and appears to be distinct from the known lipid products of PMN. The data suggest that PMN control their adhesivity through a novel lipid that may act as an allosteric activator of leukocyte integrins.

# *Chapter 1 - Introduction*

## Inflammation

Inflammation occurs in tissues in response to infection or chemical or physical trauma. Massive influx of inflammatory cells, including the polymorphonuclear leukocyte (PMN) is a hallmark of the inflammatory response. In the 1st century, A.D., Cornelius Celsus noted the four cardinal signs of inflammation that result from this leukocytic infiltration as well as changes in vascular flow and permeability: *rubor* (redness), *tumor* (edema), *calor* (heat) and *dolor* (pain). A fifth clinical sign, *function laeso* (loss of function) was later added by Virchow (Cotran et al.,1989). The cells of the inflammatory response clear infectious agents or other foreign substances, eliminate or repair dead or damaged tissues and, due to their potent destructive ability, may sometimes damage nearby normal tissues, resulting in disease.

## The role of the neutrophil in inflammation

PMN play a central role in the inflammatory response (figure 1) (Malech and Gallin, 1987). At the inflammatory site, PMN release products of the oxidative burst such as hydrogen peroxide and free radicals, which attack and digest the triggering agent. PMN also release lytic and bactericidal enzymes from intracellular granules as well as mediators that recruit more PMN to the site as well as other inflammatory cells involved in later phases of the response. Finally, PMN can phagocytose bacteria and cells, killing them by

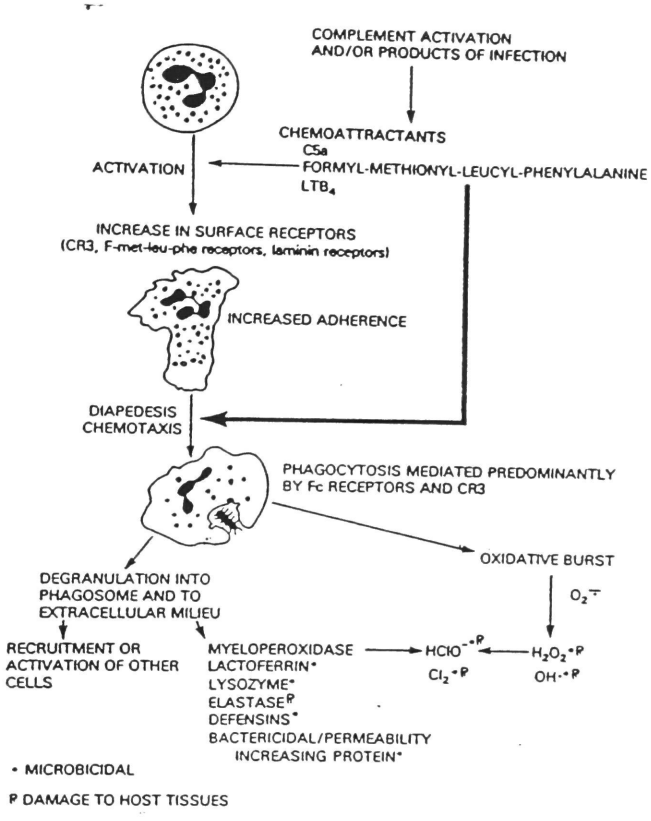



**Figure 1. Neutrophil responses to infection or other causes of inflammation.** (from Malech and Gallin, 1987)

release of intracellular mediators into the phagosome. All of these vital functions of PMN require the cell to leave the vasculature to reach the site of inflammation.

**Interactions of the neutrophil with the endothelium**

The migration of leukocytes into tissues, also referred to as diapedesis, is a key event in the inflammatory response. Normally, PMN circulate freely in the blood and do not interact with the endothelial cells (EC) lining the vessel. In response to a wide range of activators and chemotactic factors, PMN become adherent to EC and migrate within seconds (average 56 seconds, range of 15 to 150 seconds) out of the bloodstream and into the tissue (Huang et al.,1988).

The process of diapedesis begins with margination of the neutrophil: the cells leave the central stream of flowing blood in a postcapillary venule and begin rolling along on the endothelium. Margination occurs within minutes of injury to adjacent tissue, as determined using intravital microscopy (Cohnheim, 1889). The postcapillary venule is the predominant site of diapedesis in inflammation; in a healthy individual it is devoid of marginating cells (Fiebig et al.,1991). Margination in the postcapillary venule is different from the "marginated pool" of approximately 50% of PMN that remain in capillary beds in the lungs and tissues, entering the circulation in response to exercise or adrenaline.

As the inflammatory reaction progresses, the round rolling PMN accumulate on the endothelium and come to a halt, at which point they

undergo a marked change in shape, flattening against the vessel wall and extending pseudopods into the intercellular junctions of EC (Marchesi, 1961).

**Molecular events that regulate diapedesis**

Both vascular changes and intercellular adhesion interactions contribute to the process of extravasation of leukocytes. In normal conditions of blood flow, hydrodynamic forces keep the leukocytes in the center of the stream (Goldsmith and Spain, 1984; Nobis et al.,1985). In inflammation, release of mediators leads to vasodilation and a slowing of blood flow and an increase in permeability. These factors act to force leukocytes to the edges of the flow, near the vessel walls. By Pouseuille's law flow velocity is slower at the sides than at the center of the stream, and thus a torque force is exerted on the round leukocyte, contibuting to the rolling observed (Chien, 1982).

Adhesion of PMN to EC at sites of tissue injury or infection appears to occur in two phases. PMN and endothelium initially increase their adhesiveness within minutes, in a process independent of protein synthesis. Then, over 1-2 hours from the onset of inflammation, new adhesion molecules are synthesized and expressed on EC which can bind resting or activated PMN (Osborn, 1990).

**Adhesion molecules involved in diapedesis**

Activation of cell adhesion molecules already present on the endothelial cell surface contributes to the first phase of increased adhesiveness that leads to extravasation. The best candidate for the molecule involved is GMP-140 (also known as PADGEM), a member of the LECCAM family of adhesion

molecules. LECCAMs, also known as selectins, are transmembrane proteins composed of three domains: a C-terminal region containing a variable number of short consensus repeats found in complement regulatory proteins, an epidermal growth factor-like region and an N-terminal lectin-like domain that appears to be involved in adhesion (Springer, 1990). GMP-140 binds PMN when exocytosed from platelet secretory granules (Larsen et al.,1989) and fixed PMN can bind GMP-140-coated plastic surfaces (Geng et al.,1990). GMP-140 is released from the Weibel-Palade bodies of EC in culture within 5 minutes after treatment with thrombin (Hattori et al.,1989) and mAb to GMP-140 block binding of resting PMN to EC stimulated with histamine for 30 minutes (Geng et al.,1990). These data imply that resting PMN could be recruited within a few minutes of release of fast-acting inflammatory mediators such as histamine, and the highly responsive PMN could then be activated by subsequent mediators.

Seconds after exposure to a variety of substances, including TNFα, leukotrienes, platelet activating factor and complement fragment C5a, PMN exhibit a marked increase in adhesivity for EC and other substrates. This protein synthesis-independent increase in adhesion is due primarily to transient activation of the leukocyte integrins LFA-1 and CR3 (figure 2). Several lines of evidence suggest a key role for the leukocyte integrins in diapedesis. A variety of studies have demonstrated that antibodies against the CD18 molecule block the inflammatory process both in in vitro and animal model systems (Hernandez et al.,1987; Simpson et al.,1988; Vedder et al.,1988;

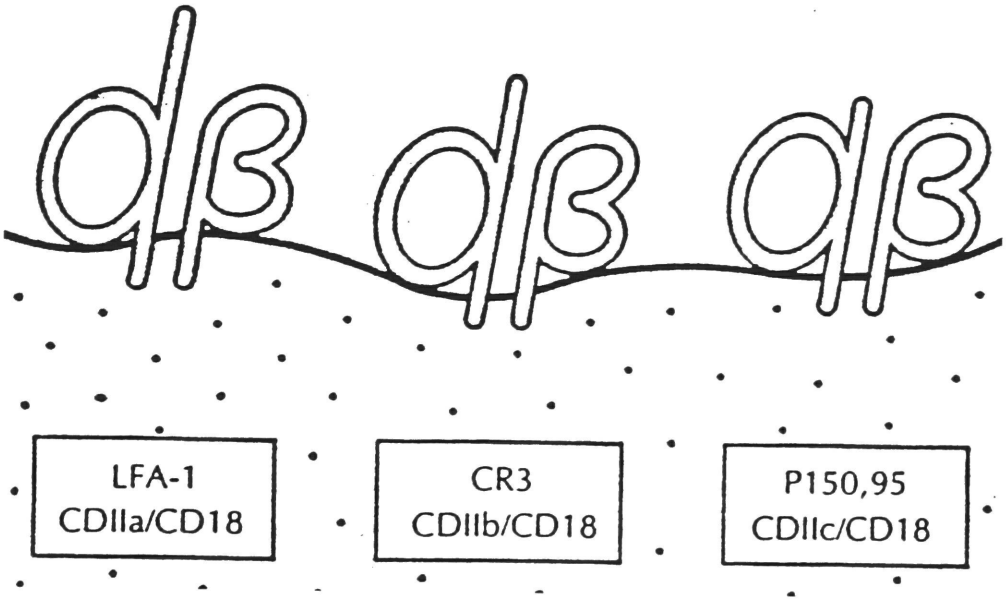



**Figure 2. Schematic representation of the three members of the leukocyte integrin family.**
Leukocytes integrins, also known as the CD11/CD18 family, are αβ heterodimers. The α chains of the family are represented by the corresponding character and illustrate the differences in molecular weight between CD11a, CD11b and CD11c (190 kD, 185 kD abd 150 kD, respectively). The β chains (CD18) are identical and have a molecular weight of 95 kD. Both the α and β chains are transmembrane proteins with small cytoplasmic domains.
(from Detmers and Wright, 1988)

Tuomanen et al.,1989). Also, patients with a disease known as leukocyte adhesion deficiency (LAD) (Anderson and Springer, 1987; Todd and Freyer, 1988) have a defect in the gene encoding the CD18 molecule which prevents the expression of the leukocyte integrins. As a result, the patients suffer from recurrent life-threatening infections with gram-positive, gram-negative and fungal pathogens, and die at a young age. They exhibit extreme leukocytosis and fail to form pus at sites of infection. In vitro experiments show PMN from LAD patients are unable to adhere to C3bi-coated particles (Wright et al.,1989), to protein-coated glass or plastic surfaces (Anderson et al.,1984) and to endothelial cells (Harlan et al.,1985). The susceptibility of LAD patients to infection is due to their inability to bind and ingest opsonized pathogens and their failure to recruit cells to inflammatory sites. Leukocytosis results from the failure of LAD phagocytes to extravasate, due to the inability to bind to EC.

Leukocyte integrins are a subfamily of a large group of receptors termed integrins (Table I)(Hynes, 1987; Albelda and Buck, 1990). Integrins are all $\alpha\beta$ heterodimers involved in cell-cell or cell-extracellular matrix adhesion events. Most integrins can be categorized into three subfamilies, determined by whether they contain $\beta_1$, $\beta_2$ or $\beta_3$ respectively, although new $\alpha$'s and $\beta$'s that fall outside these categories are being discovered. Every integrin requires warm temperatures and divalent cations for binding and some recognize a common tripeptide sequence, arg-gly-asp (RGD) on their ligands. The leukocyte integrins are a group of three receptors that share a common $\beta$ chain

*The integrin receptor family*

| β Subfamilies | | α Subunits | Ligands |
|---|---|---|---|
| | | $\alpha_1$ = VLA-1 (CD49a) | LM (COL) |
| $\beta_1$ | Chicken integrin band 3 | $\alpha_2$ = VLA-2 (CD49b) platelet GP Ia | COL (LM) |
| | Fibronectin receptor β | $\alpha_3$ = VLA-3 (CD49c) | FN, LM, COL |
| | VLA - β (CD29) | $\alpha_4$ = VLA-4 (CD49d) | FN (alt) |
| | Platelet glycoprotein IIa | | V-CAM |
| | (refs 1-4,8,15, 20-24) | $\alpha_5$ = VLA-5 (CD49e) platelet GP Ic | FN |
| | | $\alpha_6$ = VLA-6 (CD49f) platelet GP Ic | LM |
| | | $\alpha_v$ = CD51 | FN |
| $\beta_2$ | LFA-1/Mac-1/p150,95 β | $\alpha_L$ = LFA-1 (CD 11a) | I-CAM |
| | (CD18) | $\alpha_M$ = MAC-1 (CD 11b) | C3bi, FB |
| | (refs 1, 2, 26, 27, 44) | $\alpha_x$ = α chain of p150,95 (CD11c) | C3bi? |
| $\beta_3$ | Platelet glycoprotein IIIA | $\alpha_{IIb}$ = platelet GPIIb (CD41) | FB, FN, VN, VWF |
| | Vitronectin receptor β (CD61) | $\alpha_v$ = vitronectin receptor (CD51) | VN, FB, VWF, TSP |
| | (ref 3, 7) | | |
| $\beta_4$ | (ref 14) | $\alpha_6$ (CD49f/CD?) [on epithelial cells] | LM? |
| $\beta_5$ | (ref 6) | $\alpha_V$ (CD51/CD?) [on epithelial cells and some carcinomas] | VN, FN (Not FB) |

*Abbreviations: LM, laminin; COL, collagen; FN; fibronectin; alt, alternatively spliced region; ic3b, breakdown product of the third component of complement; FB, fibrogen, VN, vitronectin; VWF, von Willebrand's factor; TSP, thrombospondin.

**Table I.** (from Albelda and Buck, 1990)

($\beta_2$ or CD18) and highly homologous, but distinct, $\alpha$ chains (CD11a, CD11b and CD11c, figure 2). The differences among the $\alpha$ chains confer distinct ligand binding specificities to the three members of this receptor subfamily.

The second pathway for adhesion of PMN to EC recruits PMN to tissues within a few hours of the original inflammatory stimulus. Inflammatory cytokines such as IL1 and TNF$\alpha$ induce expression of two adhesion molecules on EC that bind PMN, ELAM-1 and ICAM-1 (Pober et al.,1986; Bevilacqua et al.,1987; Dustin and Springer, 1988). ICAM-1 is a member of the Ig superfamily of adhesion molecules that can bind LFA-1 and CR3 on PMN (Marlin and Springer, 1987; Staunton et al.,1988; Diamond et al.,1990). ELAM-1 is a LECCAM that can bind a ligand on the surface of PMN and act as a "tethered chemoattractant", activating CR3 on PMN (Lo et al.,1991). Under in vitro conditions of shear stress simulating venous flow conditions, integrin-independent adhesion of PMN to IL1-treated EC withstands higher shear stresses than integrin mediated adhesion (Lawrence et al.,1990; Lawrence and Springer, 1991). LAD or anti-CD18-treated cells, that bind to EC through adhesion mechanisms other than integrins, fail to undergo transendothelial migration, consistent with the absence of neutrophil extravasation in leukocyte adhesion deficiency (Smith et al.,1988). These data suggest the existence of an adhesion cascade, in which LECCAMs capture PMN and trigger integrin-mediated transmigration.

**CR3 on PMN is regulated**

The focus of the work presented here is a particular leukocyte integrin

known as complement receptor type 3, or CR3 (also known as Mo1, Mac-1 or CD11b/CD18). CR3 binds several ligands, including fibrinogen, C3bi (a cleavage product of the third component of complement), various microbial surface antigens, and ICAM-1 and an as yet uncharacterized ligand on EC (Wright et al.,1990; Diamond et al.,1990); the latter interactions occur in diapedesis.

In the absence of an inflammatory stimulus, the adhesive mechanisms involved in diapedesis are not needed. These adhesion interactions must therefore be regulated. As noted earlier, the endothelium controls ELAM-1-dependent adhesion by fusing Weibel-Palade bodies with the plasma membrane in response to inflammatory stimuli (Hattori et al.,1989). In contrast, CR3 is not regulated by PMN simply by changes in levels of expression (Buyon et al.,1988; Philips et al.,1988; Vedder and Harlan, 1988; Lo et al.,1989). In circulating, resting PMN, CR3 binds poorly to EC3bi (Wright and Meyer, 1986) fibrinogen-coated substrates (Wright et al.,1988) or endothelium (Lo et al.,1989). In order for CR3 to play a role in diapedesis, it must be activated. Circulating PMN exposed to inflammatory mediators increase the adhesivity of CR3, bind to EC via CR3 and then must release their hold on the EC in order to make their way through the vessel wall.

The interaction of CR3 with its ligands is regulated (figure 3) (Wright and Meyer, 1986; Lo et al.,1989). Resting PMN bind C3bi poorly, but when PMN are treated with an agonist such as phorbol myristate acetate (PMA), there is a rapid, transient increase in the extent of C3bi binding (left panel).

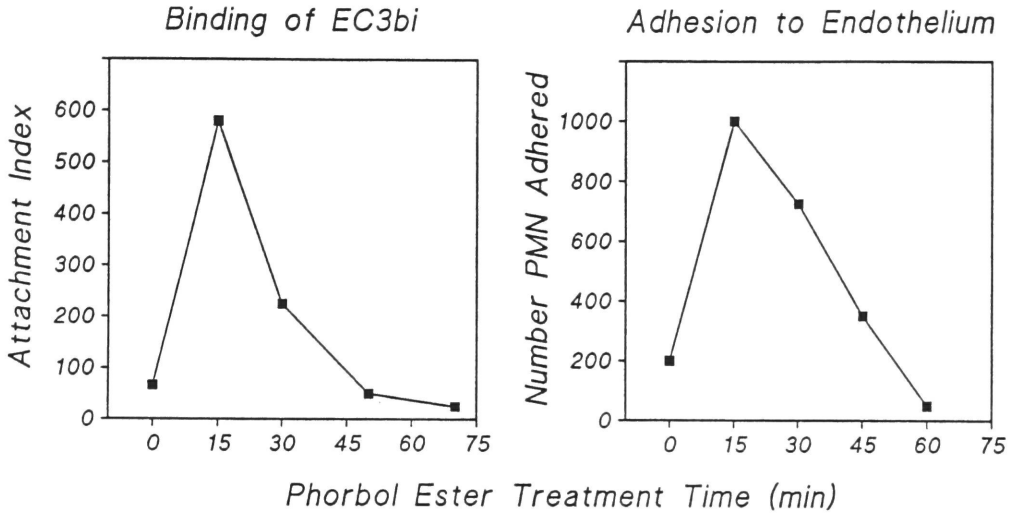



**Figure 3. CR3 binds ligand in a regulated manner.**
PMN treated with phorbol esters exhibit a transient rise in CR3-mediated binding to C3bi (left panel) and endothelium (right panel).
(from Wright and Meyer, 1986; Lo et al., 1989a)

This transient increase in CR3 binding in PMN treated with phorbol ester is true for another ligand of CR3, the endothelium (right panel). The transient nature of this response to agonist appears necessary to allow the cell to cross the endothelial barrier. A moving cell must adhere to the substrate at the leading edge but also needs to detach its uropod for locomotion to occur. The loss of avidity of CR3 may allow detachment of the uropod and reutilization of receptors for a new round of adhesion.

The ability of CR3 to bind fibrinogen is also transiently enhanced by treatment with agonists (see chapter 5). Other agonists of PMN, such as tumor necrosis factor (TNF), C5a, formylated peptides, and NAP-1/IL8, can also trigger a transient increase in the ability of CR3 to bind its ligands (Lo et al.,1989; Detmers et al.,1990; SD Wright, unpublished observations). The classes of molecules that can affect CR3 avidity on PMN has recently been found to include surface-bound agonists. ELAM-1 on cytokine-treated EC can increase CR3 avidity in PMN in contact with the endothelium (Lo et al.,1991), and binding of LPS/LBP complexes to CD14 on the PMN can also trigger increased CR3 binding to C3bi-coated particles (Wright et al.,1991).

Agonists that enhance the avidity of CR3 on PMN do cause a 2-3 fold increase in CR3 expression due to exocytosis of specific granules, but this increase in receptor expression is not the only mechanism contributing to the change in CR3 function (5-10 fold) that occurs in response to these agonists. PMN treated with the anion channel-blocking agent DIDS (4,4'-diisothiocyanostilbene-2,2'-disulfonic acid) do not degranulate in response to

normal stimuli. These cells, however, are capable of binding to cultured endothelial cells in response to PMA, A23187, or fMLP in the absence of upregulation of receptors on the cell surface (Vedder and Harlan, 1988). In addition, cytoplasts, which are enucleated neutrophils depleted of specific granules containing CR3, are also able to bind to endothelium (Vedder and Harlan, 1988; Lo et al.,1989) or EC3bi (SD Wright, unpublished) although there is no change in the expression of the receptor after stimulation with PMA, A23187 or fMLP (Vedder and Harlan, 1988; Philips et al.,1988). Moreover, blockade of cell surface CR3 with monoclonal antibody blocks CD18-dependent aggregation of PMN despite the recruitment of new unblocked receptors from intracellular pools (Buyon et al.,1988). Together these data suggest that regulation of CR3 adhesivity is a qualitative rather than quantitative change in the receptor. The molecular basis of this change in CR3 adhesivity has not been elucidated.

The work presented here was undertaken to determine the molecular mechanism by which CR3 function is regulated.

# *Chapter 2 - Materials and Methods*

**Buffers**

Phosphate buffered saline (PBS) contains 137 mM NaCl, 2.7 mM KCl, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 8 mM phosphate pH 7.4; PD is PBS deficient in $Ca^{++}$ and $Mg^{++}$ ions; HAP is PBS containing 0.5 mg/ml human serum albumin, 3 mM glucose and 0.3 U/ml aprotinin; $DGVB^{++}$ is 2.5 mM veronal buffer pH 7.5 containing 75 mM NaCl, 2.5% glucose, 0.05% gelatin, 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$.

**Monoclonal antibodies**

mAbs OKM1 (IgG2b) and OKM10 (IgG2b), directed against the α chain (CD11b) of CR3 (Wright et al.,1983) were a gift of Dr. G. Goldstein (Ortho Pharmaceutical, Raritan, NJ); mAb 44 (clone aacf) against CD11b (Dana et al.,1986), was provided by Dr. R. F. Todd III (U of Michigan, Ann Arbor, MI); mAb TS1/22 (IgG1) directed against the α chain (CD11a) of LFA-1 (Sanchez-Madrid et al.,1982) was a gift of Dr. T. Springer (Dana-Farber Cancer Institute, Boston, MA); mAb LeuM5 (IgG2b) directed against the α chain (CD11c) of p150,95 (Lanier et al.,1985) was a gift of Dr. L. Lanier (Becton Dickinson & Co., Mountain View, CA); AIIB2 against integrin $\beta_1$ chain (Brown et al.,1989) was provided by Dr. C. Damsky (UCSF, San Francisco, CA); mAb IB4 (IgG2a) directed against the β chain (CD18) of CR3, LFA-1 and p150,95 was as described (Wright et al.,1983); mAb 3G8 (IgG1) directed against the low avidity Fcγ receptor of neutrophils (FcRIII, CD16) was as described (Fleit et

al.,1982); W6/32 against class I histocompatability antigens (Barnstable et al.,1978) was from the American Tissue Type Culture Collection (Rockville, MD); LB-2 against ICAM-1 (Patarroyo et al.,1987) was a gift of Dr. E. Clark (Seattle, WA); C3-9 directed against the third component of complement (Hack et al.,1988) was a gift of Dr. E. Hack (University of Amsterdam, The Netherlands); FITC-conjugated PAC-1 against activated gpIIb/IIIa (Shattil et al.,1985) was kindly provided by Dr. S. Shattil (U of Pennsylvania, Philadelphia, PA) and FITC-conjugated goat anti-mouse IgG was from HyClone Laboratories.

**Cells**

PMN were purified from fresh human blood on Ficoll-Hypaque gradients (English and Anderson, 1974) and suspended in HAP buffer; THP-1A cells, a monocytoid cell line, are as described (Auwerx, 1991), SCHU cells are an EBV transformed B lymphoblastoid cell line kindly provided by Dr. S. Y. Yang (Memorial Sloan-Kettering, NYC) that grow in suspension in RPMI with 10% fetal calf serum and 0.1% penicillin/streptomycin; human monocytes and lymphocytes were purified from buffy coats (Greater New York Blood Center) on Percoll gradients. Monocytes were cultured in 12.5% human serum in Teflon beakers as previously described (Wright and Silverstein, 1982). Upon cultivation for 4-10 days, the cells matured into macrophages. Alternatively, lymphocytes were purified from the mononuclear cell band on Ficoll-Hypaque gradients by removing monocytes with a plastic adherence step (2 hours, 37°C).

Extraction of IMF-1

A pellet of resting or stimulated whole PMN was resuspended by stirring, vortexing and, on occasion, sonication, into chloroform:methanol:water=10:10:1, at a concentration of at most 2.5 x $10^8$ PMN/ml. After stirring for a minimum of 36 hours, with at least 3 solvent changes, supernatants were pooled and dried down under vacuum in a rotary evaporator. The dried extract was resuspended in 3 bed volumes of chloroform:methanol:water = 30:60:8, and passed through a DEAE-Sephadex (Pharmacia) column. A column of 2-20 ml in volume was used for starting material from $10^9$ - $10^{10}$ cells, respectively.The column was then washed with 6 bed volumes of solvent and eluted with at least 3 bed volumes of chloroform:methanol:0.8 M sodium acetate = 30:60:8. The eluate was dried down under vacuum and resuspended in distilled water to a volume of up to 1 ml for $10^8$ PMN of starting material. A Sep-Pak C18 (Waters) cartridge or a hand-packed column of about 10 ml of the Sep-Pak sorbent was used to desalt material from $10^9$ - $10^{10}$ cells, respectively. The $C_{18}$ column was prewashed in methanol, chloroform:methanol = 2:1, and methanol:1.6 M sodium acetate = 1:1 before sample was applied in at least 3 bed volumes of water. Salt was washed out by extensive rinsing of the column with water (>6 bed volumes) and the lipid extract was eluted with 2 bed volumes of methanol and at least 6 bed volumes of chloroform:methanol = 1:1. The eluted material was dried down and resuspended either in chloroform:methanol = 2:1 for storage as a stock of partially purified IMF-1 or was additionally fractionated

in a Folch partitioning step, in which IMF-1 activity separates to the saline upper phase in equilibrium with a lower phase of chloroform:methanol = 2:1. Unless otherwise stated, the IMF-1 used in these experiments was taken from the partially purified stock resulting from the Sep Pak step. IMF-1, other lipids, and agonists were all dissolved in PBS for application to cells or isolated receptor. Control lipids represent the lipids extracted from resting PMN and were used at comparable or greater numbers of cell equivalents of lipid to the amount of IMF-1 used in the same experiment.

Less than 0.1% of IMF-1 activity was found in the flowthrough from the DEAE and Sep-Pak columns. However, up to 50% of IMF-1 activity was lost in the Folch cut. Since the activity was not recovered in the organic phase, it is possible that the procedure destroys IMF-1 activity and therefore it is usually ommitted from isolations of IMF-1. Total yield of IMF-1 ranged from 10-33% of the material initially found in the chloroform/methanol extract of the cell pellet.

**Rosetting assay**

The ability of CR3 on PMN to bind ligand is measured using the rosetting assay, as previously described (Wright and Meyer, 1986). Briefly, lipid extract or agonists were added to adherent PMN ($2 \times 10^6$ /ml) at 37°C for varying times, and the cells were washed. Under standard conditions for assaying IMF-1 activity, lipids or lipid extracts were incubated with PMN for 15 minutes. Sheep erythrocytes bearing covalently attached C3bi (EC3bi) or some other ligand for CR3, such as lipid IVa, a biosynthetic precursor of LPS

(Wright and Silverstein, 1982; Wright and Jong, 1986), were then incubated with the PMN for 15 minutes at 37°C. Unattached erythrocytes were washed away and bound EC3bi were scored by phase contrast microscopy. The attachment index is the number of erythrocytes bound per 100 PMN. Rosetting was blocked by addition of mAbs (10 μg/ml) against CR3 or C3bi.

A unit of IMF-1 activity is defined as the amount of IMF-1 needed to half-maximally activate $1.2 \times 10^5$ PMN in this rosetting assay. $10^7$ PMN yield 3-10 units of activity in a typical IMF-1 extraction. This means that extract from 8-25 PMN can half-maximally activate a single cell in a volume of one nanoliter. Half-maximal binding is achieved with an IMF-1 concentration of 8.3 U/ml.

Lipid IVa, used to make IVa-coated erythrocytes, was a generous gift of Dr. C. R. H. Raetz (Merck, Sharp and Dohme Research Laboratories, Rahway, NJ). Isoprenoids tested for IMF-1 activity in the rosetting assay were kindly provided by Dr. P. Low (Stockholm University, Sweden).

**Affinity purification of CR3**

The purification of CR3 will be described in detail elsewhere (JAG Van Strijp and SD Wright, manuscript in preparation). Briefly, C3bi from human serum was covalently bound to Sepharose beads through the action of the alternative pathway of complement activation. SDS-PAGE and immunoblotting confirmed that >95% of the deposited protein on the beads was C3bi. $10^8$ PMN were lysed in 2.5% octylglucoside, 20mM HEPES buffer pH 7.4

containing 50mM NaCl, 1mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 1 μg/ml each of the protease inhibitors antipain, benzamidine, chymostatin, leupeptin, and pepstatin A, 0.24 U/ml of aprotinin and 2 mM PMSF (lysis buffer). The lysate was cleared by centrifugation and incubated with C3bi-coupled Sepharose for 3 minutes at room temperature. Beads were washed extensively with lysis buffer and CR3 was eluted with 10 mM EDTA. Purity of extracted CR3 was assessed by SDS-PAGE and silver staining.

**Assay of purified CR3**

CR3 eluted from C3bi-Sepharose was diluted into lysis buffer without detergent and Terasaki plate wells were coated with purified CR3 for one hour at 4°C. Wells were washed extensively with PBS. The number of available CR3 epitopes per $\mu m^2$ of well surface was determined by using iodinated monoclonal antibody 44a, against CD11b. Various amounts of purified CR3 adsorbed to wells were washed with Dulbecco's PBS and IMF-1 was added in PBS. After 30 minutes incubation at room temperature in the presence or absence of IMF-1, wells were washed with PBS and EC3bi were added at $10^8$/ml in the presence or absence of mAbs 44a or LeuM5 (50 μg/ml) and incubated for 20 minutes at room temperature. The plate was inverted to remove unattached erythrocytes and washed. Bound erythrocytes were scored by phase contrast microscopy.

**Binding of PMN to fibrinogen-coated surfaces**

Surfaces were coated for 60 minutes with 1 mg/ml fibrinogen (depleted of plasminogen, a generous gift of Dr. L. Ossowski, Mt. Sinai Medical Center,

NYC) in PD. PMN were allowed to adhere to fibrinogen-coated surfaces at 37°C for varying times in the presence or absence of 42 U/ml of IMF-1, partially purified from fNLLP treated cells.

**Binding of PMN to endothelium**

PMN ($2 \times 10^6$/ml) were treated with or without IMF-1 (42 U/ml, isolated from cells treated with fNLLP) for 15 minutes or for varying times at 37°C, washed, and allowed to bind to unstimulated confluent human umbilical vein endothelial monolayers for 15 minutes at 37°C, in the presence or absence of mAbs (10 μg/ml). Nonadherent cells were then washed away and bound cells were quantitated using phase contrast microscopy.

**Aggregation of lymphocytes**

Lymphocytes were purified on Percoll gradients as described (33), and contaminating monocytes were removed by plastic adherence. Cells ($10^6$/ml) were then added to Terasaki plate wells and PMA (100 ng/ml), IMF-1 (42 U/ml) or lipids extracted from resting PMN ("control lipids", extracted from 3 times as many cells as the IMF-1) were added in the presence or absence of antibodies (10 μg/ml). Cells were incubated 30 minutes at 37°C and aggregation was determined by phase-contrast microscopy and reported as: %aggregation = [1-(free cells per $mm^2$ in test well/free cells per $mm^2$ in control untreated wells)] x 100.

**Platelet aggregation and flow cytometry**

Platelets were purified as described (Silverstein and Nachman, 1987). Briefly, whole blood was centrifuged at 180g and the platelet fractions were gel

filtered on Sepharose 2B. Aggregation of a solution of approximately 2 x $10^8$ platelets/ml in modified Tyrode's buffer containing up to 200 µg/ml fibrinogen and 1 mM $MgCl_2$ was monitored in a Payton platelet aggregometer set at 37°C under stirring conditions. IMF-1 up to 100 U/ml was tested, and compared to 3 U/ml thrombin as control. As a control, aggregation of platelets to very low concentrations of thrombin (0.018 U/ml) was shown to be fibrinogen dependent. For flow cytometry studies, $10^6$ platelets were incubated for 15' at 20°C with FITC-conjugated anti-$\alpha_{IIb}$ (PAC1, 40 µg/ml) in the presence of IMF-1 (50 U/ml), lipid extracts lacking IMF-1, or thrombin (0.2 U/ml).

**Adhesion of lymphocytes to fibronectin**

Terasaki wells were coated with fibronectin (100 µg/ml, Greater New York Blood Center) for 60', 20°C, washed and cells were added for 30', 0°C, in the presence or absence of Ab (10 µg/ml or neat hybridoma supernatant). PMA (30 ng/ml) or IMF-1 (42 U/ml) were added for 10', 37°C. Nonadherent cells were washed away and remaining cells counted.

**Reverse phase chromatography**

A Waters µBondaPak $C_{18}$ reverse phase column (10 µm particle size, 3.9mm x 150mm) was loaded with IMF-1 (50 units, partially purified from fNLLP treated cells), $LTB_4$ (5 ng, and 0.5µCi) or $LXA_4$ (5 µg) and eluted with a 1% per minute gradient of water to acetonitrile. IMF-1 elution was monitored by testing fractions in the rosetting assay, $LTB_4$ by cpm per fraction and $LXA_4$ by absorbance at 254 nm. IMF-1 was dried down and resuspended in water and

loaded after a 5′ spin at 10,000 g to remove insoluble material. A rosetting assay on the material loaded revealed no depletion of IMF-1 activity upon removal of particulates.

**Size exclusion chromatography**

IMF-1 (partially purified from fNLLP treated cells, or derived from column fractions of a previous run) was separated on a TSK G2000HXL size exclusion chromatography column (Supelco) run in tetrahydrofuran. IMF-1 was dried down and resuspended into tetrahydrofuran, and loaded after a 5′ spin at 10,000 g to remove insoluble material. This procedure did not deplete the loaded material of IMF-1 activity, as determined by rosetting assay comparison of the suspension before and after spinning. Elution volumes of polystyrene molecular weight standards in the range of 1000 - 180 daltons were used to generate a calibration curve. Linear regression yields a standard curve of $y = -1.278x + 3.302$ with $r2=0.9884$. IMF-1 activity of each fraction was determined by rosetting assay.

**Inhibitor studies**

PMN treated with WEB 2086 (10 μM; 25′), indomethacin (10 μM; 100′), MK886 (50, 5, 0.5 nM; 50′), L651,392 (5, 0.5, 0.05 μM; 50′) or bromophenacyl bromide (30, 3, 0.3 μM; +/- arachidonate 10, 1, 0.1 μM; 70′) were tested for their ability to bind EC3bi in the rosetting assay with or without an incubation with PMA (30 ng/ml, 20′) or IMF-1 (42 U/ml, 20′).

**Lovastatin treatment in the rosetting assay**

Lovastatin, a competitive inhibitor of the enzyme HMGCoA reductase, was

kindly provided by Dr. Alberts (Merck Sharpe and Dohme Research Laboratories, Rahway, NJ) and was base treated to open the lactone ring as follows: 16 mg/ml lovastatin in 40% ethanol containing 60 mM NaOH was heated to 50°C for 2 hours, neutralized with HCl and diluted to a 4 mg/ml stock with H2O (final concentration of ethanol in stock was 10%). Lovastatin (40 µg/ml in PBS), was added to adherent PMN for 90 minutes at 37oC in the presence or absence of mevalonate (50 ng/ml in PBS) to overcome the block. After 10 minutes in lovastatin, C5a ($10^{-8}$M in PBS) was added and after 70 minutes in lovastatin, PMA (30 ng/ml in PBS) or IMF-1 (42 U/ml) was added. After 75 minutes in lovastatin, EC3bi were added for 15 minutes and the attachment index was measured.

**Mevalonate labeling**

500 µCi R,S-[5-$^{3}$H(N)]-Mevalonate (40 Ci/mmol, New England Nuclear) and 80 µg lovastatin were dried down in a glass 12 x 75 mm tube and 2 x $10^{8}$ PMN were added in 0.5 ml HAP buffer. After 20′ at 37°C, C5a (Dr. M. Springer, Merck, Sharp & Dohme Research Laboratories, Rahway, N.J.) was added to $10^{-8}$M final concentration. After another 1 hour and 40 minutes at 37°C, TNF was added to a final concentration of 5 x $10^{3}$ U/ml and incubated at 37°C for 15′. Cells were spun out and resuspended in about 5 ml of chloroform:methanol:water=10:10:1. After a total of 24 hours of stirring with 2 solvent changes, extracts were pooled and half was dried down and resuspended in 20% $CH_3CN$ (aq) and run on the Waters $C_{18}$ reverse phase column as described. 11.35% of the counts added were incorporated into the

cells. For the chromatography step, 10% of the counts were lost to precipitation after dissolving in 20% $CH_3CN$ (aq).

**Gas Chromatography / Mass Spectroscopy**

70,000 units of partially purified IMF-1 was purified on a $C_{18}$ Waters μBondapak reverse phase column (10μm particle size, 3.9mm x 150 mm) in a 1% per minute gradient of 20 - 60% acetonitrile (aq). Elution of IMF-1 at 47% $CH_3CN$ was verified by testing column fractions by rosetting assay. Purified IMF-1, as well as material from fractions eluting just before and after those containing IMF-1 activity, was derivatized with trimethylsilane (or deuterated TMS) using 1:1=BSTFA (bis-trimethylsilyltrifluoroacetamide, or BSTFA-d18):pyridine, 50°C, 30′ and loaded onto a capillary gas chromatography column (J&W DB-5-30W, 15m) in series with a Finnigan-MAT TSQ70B (triple quadrupole) mass spectrometer in electron impact (EI, 70ev) ionization mode. The sample was eluted from the GC column by the following temperature ramp: initial temperature 70°C hold for 2 minutes, ramp to 300°C at 10°/minute, hold for 1 minute. The mass spectrometer was scanned from 40 to 800°C every second. The samples were also run underivatized or as silyl derivatives by direct probe in both the EI and fast atom bombardment (FAB) ionization mode, but no peak corresponding to the 315 species was observed. All data was analyzed using the ICIS data system.

# *Chapter 3 - Discovery of a molecule that can activate complement receptor type 3*

## The IMF-1 hypothesis

Agonists stimulate PMN and lead to a variety of effects, including degranulation, $Ca^{+2}$ transients, generation of the oxidative burst and a transient increase in the avidity of CR3. A homologous receptor, an integrin which recognizes vitronectin ($\alpha_V\beta_3$), was found to colocalize with gangliosides $GD_2$ and $GD_3$ in adhesion plaques on the melanocyte membrane, suggesting that integrin function may be regulated by an association with lipids (Cheresh et al.,1987). The work presented here was undertaken to determine whether a lipid may be responsible for regulating the avidity of CR3. The data are consistent with the following hypothesis. PMN stimulated with agonists would rapidly produce a specific lipid, called integrin modulating factor-1, or IMF-1, from a putative precursor (figure 4). Alternatively, this lipid may be sequestered in a resting PMN and released upon activation. IMF-1 would bind directly to CR3 or perhaps alter the microenvironment of the receptors, inducing receptor clustering, resulting in an increased ability of the receptor to bind ligand. Activation of the receptor is transient: IMF-1 may be degraded by some cellular enzyme or inactivated by binding to an inhibitor, or otherwise sequestered. Thus it is proposed that the ability of CR3 to bind ligand is regulated by a lipid, IMF-1, produced by PMN upon stimulation with agonist, that acts as an allosteric activator of the receptor.

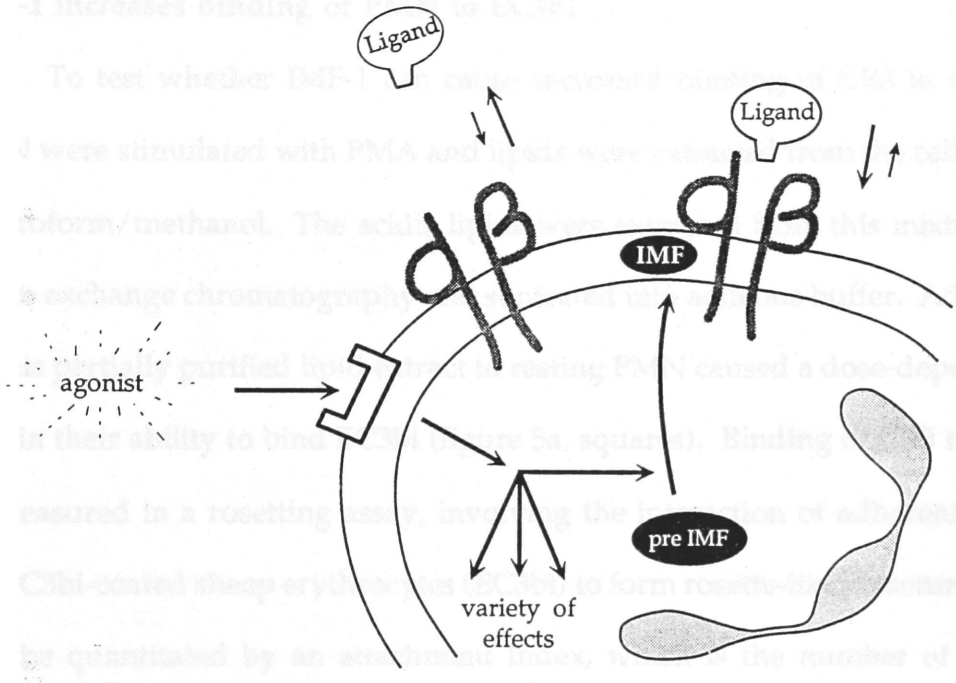



**Figure 4. The IMF-1 hypothesis.**
Agonists interact with specific surface receptors on PMN, triggering intracellular signals that lead to a variety of effects, including $Ca^{++}$ transients, generation of the oxidative burst and a transient rise in adhesivity mediated by CR3. The latter response may be due to production of a lipid, IMF-1, from a specific precursor, pre-IMF-1. When present in the cell, IMF-1 would interact directly with CR3 to increase the receptor's ability to bind ligand.

The preceding hypothesis suggests three testable predictions: 1) addition of IMF-1 to unstimulated PMN should cause an increase in CR3 binding to ligand; 2) IMF-1 should only be found in stimulated, and not resting, PMN; and 3) IMF-1 should be able to bind directly to isolated CR3 and alter its avidity for ligand.

**IMF-1 increases binding of PMN to EC3bi**

To test whether IMF-1 can cause increased binding of CR3 to ligand, PMN were stimulated with PMA and lipids were extracted from the cells with chloroform/methanol. The acidic lipids were extracted from this mixture by anion exchange chromatography and sonicated into aqueous buffer. Addition of this partially purified lipid extract to resting PMN caused a dose-dependent rise in their ability to bind EC3bi (figure 5a, squares). Binding of CR3 to C3bi is measured in a rosetting assay, involving the interaction of adherent PMN and C3bi-coated sheep erythrocytes (EC3bi) to form rosette-like structures that can be quantitated by an attachment index, which is the number of EC3bi bound per 100 PMN, as determined by phase contrast microscopy. Increased binding of PMN treated with lipid extract to EC3bi was mediated by CR3, because binding could be blocked with mAbs against CR3 $\alpha$ or $\beta$ chain (OKM10, IB4), as well as mAb against C3 (mAb C39), while a non-blocking anti-$\alpha$ chain mAb (OKM1) had no effect (Table II). The partially purified lipid extract acts specifically on CR3 because binding of erythrocytes coated with IgG, mediated by Fc receptors, was unaffected in PMN exposed to the extract (fig 5a, open triangles). These observations indicate that the partially purified

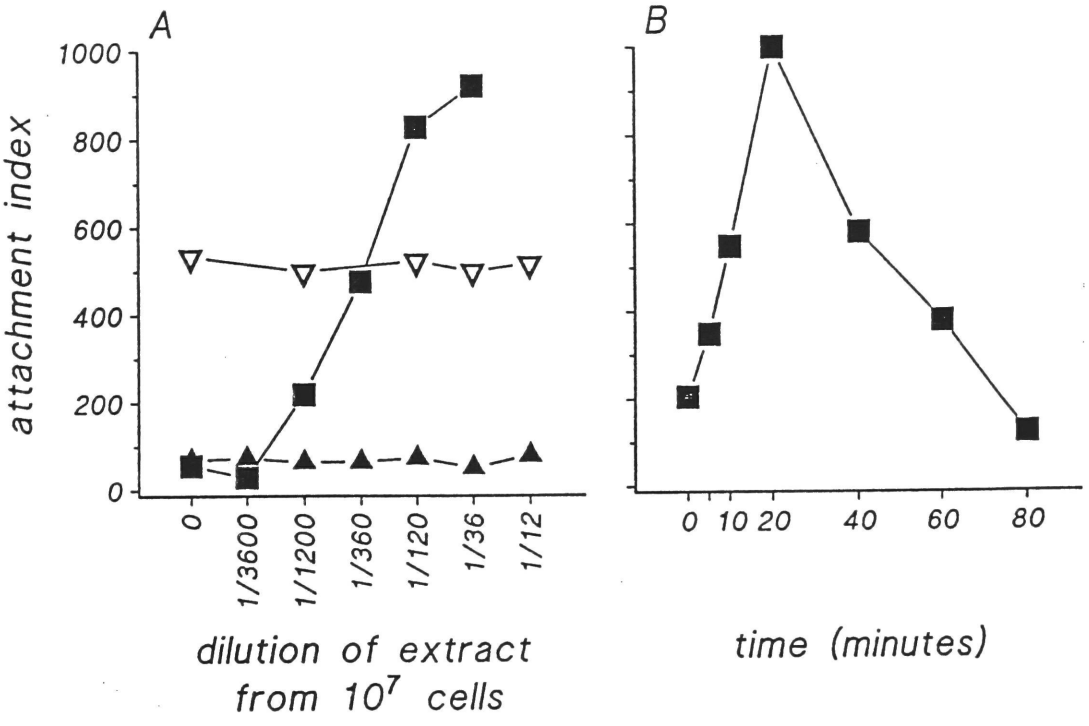



**Figure 5. IMF-1 increases CR3-mediated binding to C3bi-coated erythrocytes in a time- and dose-dependent manner**

panel A. Dose-dependence.

Partially purified IMF-1 (from cells treated with PMA, 20′) was prepared at various dilutions and applied to resting adherent PMN for 15 minutes at 37°C. The ability of IMF-1-treated cells to bind EC3bi (squares) was measured in the rosetting assay (see Materials and Methods). Lipids extracted from unstimulated PMN (solid triangles) had no effect on CR3 activity at any dilution. IMF-1 in the lipid extract had no effect on binding of erythrocytes coated with IgG (open triangles).

panel B. Time course.

Partially purified lipids from PMN treated with the chemotactic peptide fNLLP for 15′ (42 units/ml IMF-1 activity) were added to adherent PMN and after various intervals, CR3 activity was measured by the rosetting assay.

lipid extract from activated PMN contains a factor, IMF-1, that can change the binding activity of CR3. A unit of IMF-1 is defined as the amount needed to achieve half-maximal binding of EC3bi to $1.2 \times 10^5$ PMN under standard assay conditions. In this experiment, dilution of lipid extract from 107 PMN by 1/360, to a final volume of 120 µl (standard volume), yields one unit. One unit in 120 µl is equivalent to 8.3 U/ml. In the IMF-1 preparation used in figure 5, $10^7$ cells were extracted to give 3 units of IMF-1. Typically, 3-10 units of IMF-1 can be extracted from $10^7$ stimulated PMN and the IMF-1 from 8-25 stimulated PMN is sufficient to half-maximally activate a single neutrophil.

The effect of IMF-1 on CR3 was transient. Enhanced binding of ligand peaked at 20 minutes and fell in the subsequent 40 minutes (figure 5b). This decline in CR3 activity may be due to degradation, sequestration or binding of IMF-1 by an inhibitor. It is likely to be due to degradation of IMF-1, because cells that were treated with IMF-1 for 60 minutes responded to readdition of IMF-1 with kinetics and a magnitude of response similar to the initial response (see figure 26).

**Levels of IMF-1 correlate with the binding activity of CR3**

IMF-1 can be extracted from PMN only upon activation, since it was not found in lipids extracted from unstimulated PMN (figure 5a, solid triangles). The amount of IMF-1 present in cells was determined by stimulating PMN for various intervals with PMA then measuring the amount of IMF-1 in extracts of the cells (figure 6, triangles). Maximal amounts of IMF-1 were obtained after 20 minutes of stimulation, but the amount of IMF-1 in stimulated cells

*BINDING INDUCED BY IMF-1 IS INHIBITABLE BY ANTIBODIES AGAINST CR3 AND C3bi*

| agonist | Ab | Ag | binding of EC3bi (AI) |
|---|---|---|---|
| none | none | | 16 |
| IMF-1 | none | | 206 |
| IMF-1 | IB4 | CD18 | 83 |
| IMF-1 | C39 | C3 | 31 |
| IMF-1 | OKM10 | CD11b | 27 |
| IMF-1 | OKM1 | CD11b | 175 |

**Table II.** Partially purified IMF-1 (from PMA-treated cells, 42 units/ml) was added to PMN in the rosetting assay in the presence or absence of various monoclonal antibodies (10 μg/ml). This experiment is representative of three repeats.

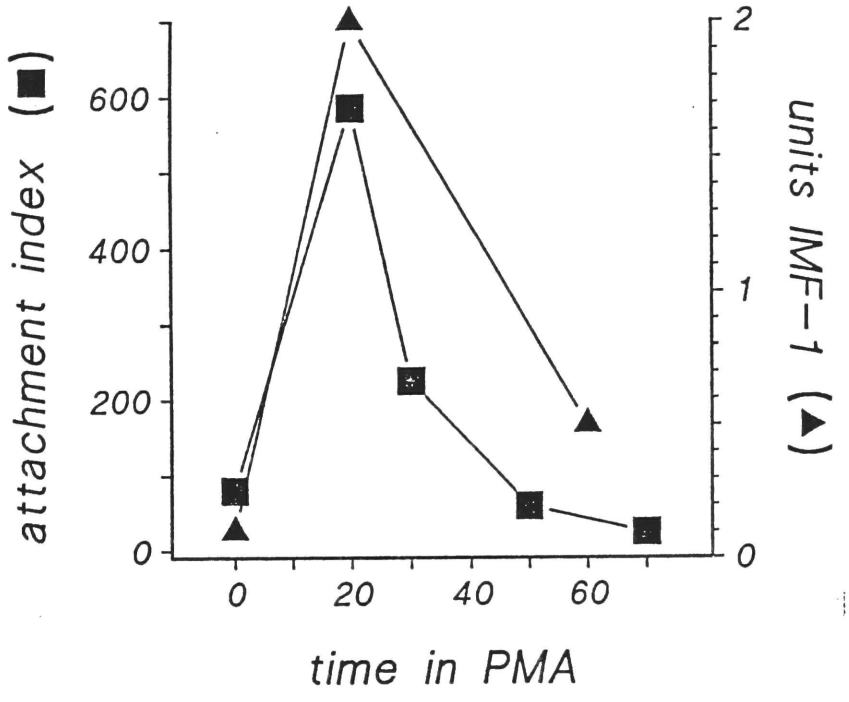



**Figure 6. IMF-1 content of PMN correlates with CR3 activity.**
Phorbol myristate acetate (PMA, 30 ng/ml) was added to resting adherent PMN for various times at 37°C, and washed away. Binding of CR3 to C3bi was then measured (squares) in the rosetting assay. A parallel set of PMA-treated PMN were extracted for lipids and the number of units of IMF-1 activity present in the extract from $10^7$ PMN was determined (triangles) by diluting the extract and measuring the amount needed to half-maximally activate $1.2 \times 10^5$ PMN to bind EC3bi.

declined to low levels by 40 minutes. The ability of CR3 to bind EC3bi also exhibits a transient rise and fall when assayed in a parallel population of cells (figure 6, squares), suggesting that the capacity of CR3 to bind ligand may be controlled by the cellular levels of IMF-1. This hypothesis was tested using additional agonists of PMN. Tumor necrosis factor (TNF, $5 \times 10^3$ U/ml, figure 7) and formyl-norleucyl-leucyl-phenylalanine (fNLLP, $5 \times 10^{-8}$ M, figure 8) each induced a transient rise in CR3 activity, and each caused a proportional rise in the amount of IMF-1 extractable from the cells. These experiments also revealed that IMF-1 could be extracted from PMN as quickly as 30 seconds after stimulation with agonist and that at all times less than 2% of the IMF-1 could be found in the overlying medium (figure 7). Thus it appears that IMF-1 remains in the cells that produce it and that it functions as an endogenous regulator of cell function. IMF-1 derived from a mixture of cells treated with PMA, TNF, fNLLP or platelet activating factor (PAF, $10^{-7}$ M, 15′) migrates as a single peak of activity on reverse phase chromatography and thus was considered to be the same molecule and used interchangeably in the experiments reported below.

**IMF-1 interacts directly with purified CR3**

CR3 was purified from a lysate of resting PMN by affinity chromatography on C3bi-coated Sepharose beads. CR3 was eluted with EDTA, the purity of the extracted CR3 was assessed by SDS-PAGE and silver staining (figure 9), and then CR3 was adsorbed to culture wells. The resulting CR3-coated surfaces bound EC3bi very poorly, but preincubation of the

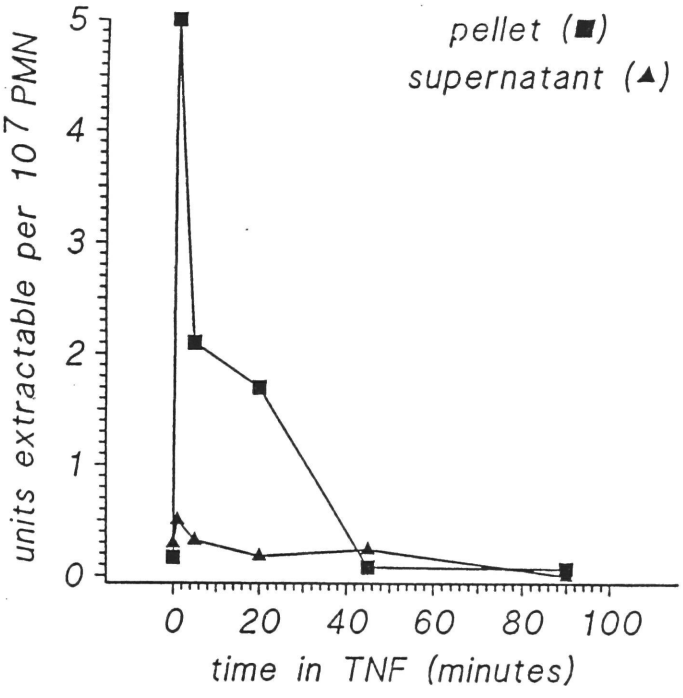



**Figure 7. The majority of IMF-1 produced by activated PMN is cell associated.**
PMN were exposed to TNF ($5 \times 10^3$ u/ml) for varying times and spun down. Pellets were extracted with chloroform:methanol:water = 10:10:1 and supernatants were dried down in a rotary evaporator and resuspended into chloroform:methanol = 2:1. The extracts were then tested for IMF-1 activity by rosetting assay.

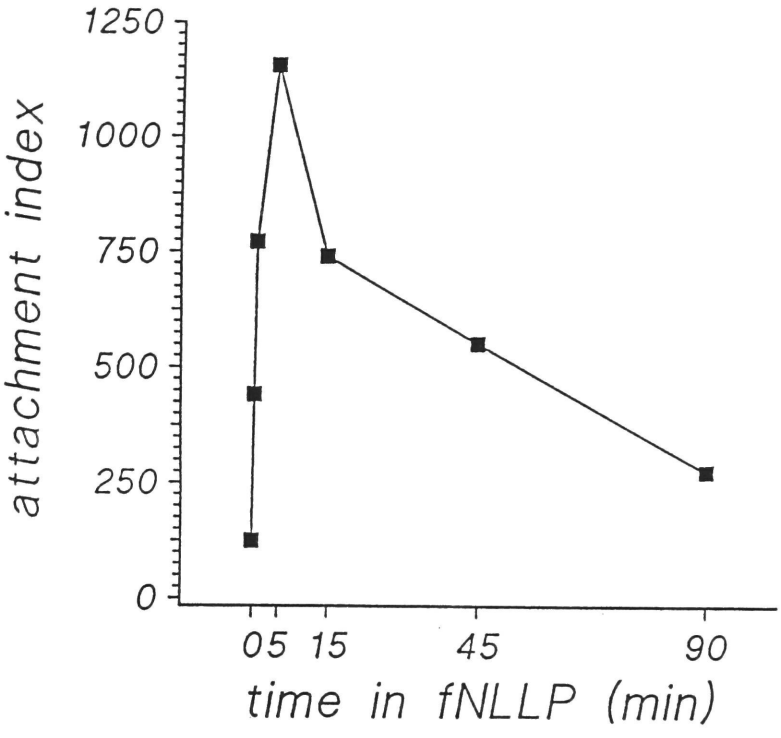
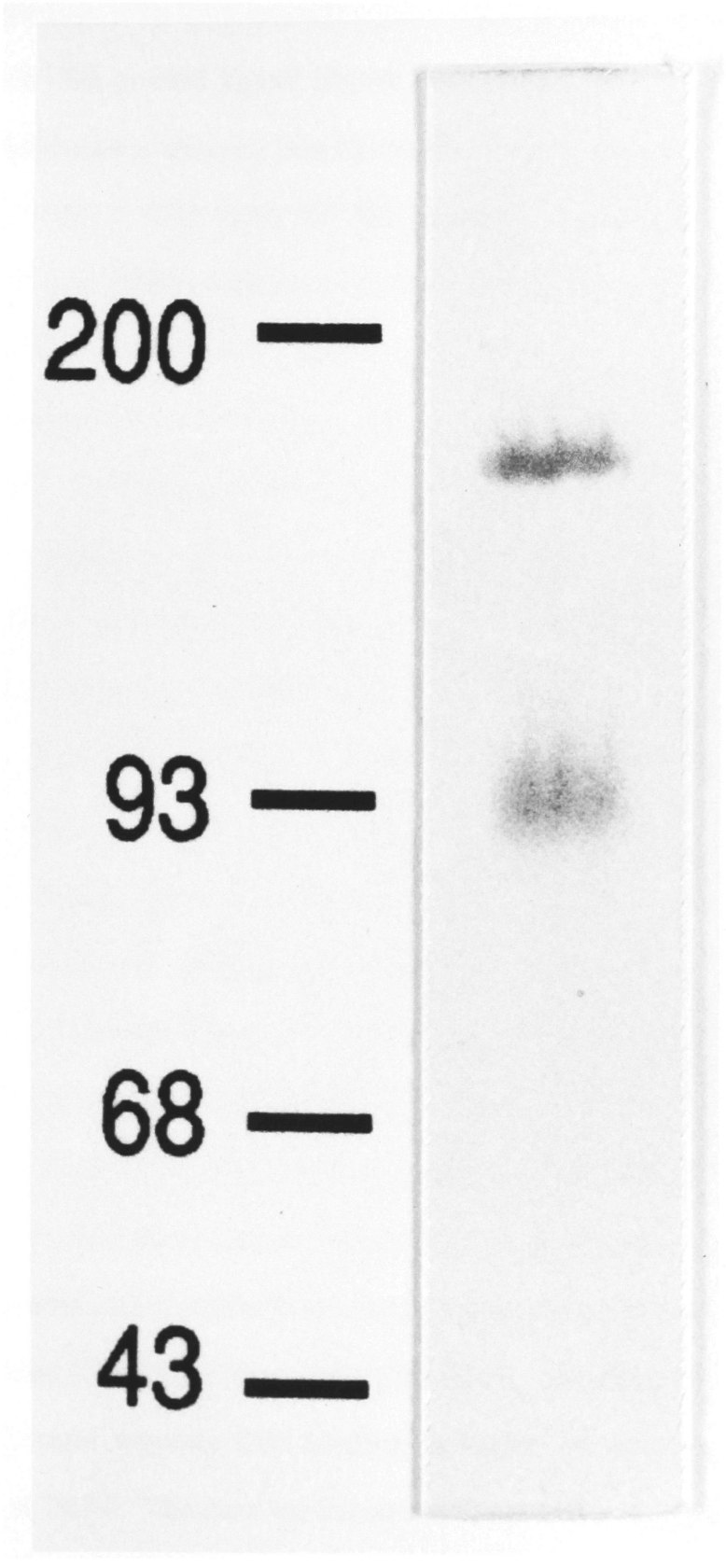



**Figure 8. IMF-1 can be produced by PMN within 30 seconds of stimulation.** PMN were exposed to fNLLP ($5 \times 10^{-8}$ M) for varying times and spun down. Pellets were extracted with chloroform:methanol:water = 10:10:1 and supernatants were dried down in a rotary evaporator and resuspended into chloroform:methanol = 2:1. The extracts were then tested for IMF-1 activity by rosetting assay.

**Figure 9. SDS-PAGE of purified CR3.**
CR3 was purified from resting PMN by affinity chromatography and run on SDS-PAGE and silverstained. The two bands
correspond to the α chain at 185 kD and the β chain at 95 kD.


200
93
68
43

adsorbed receptor with lipid extracts containing IMF-1 dramatically enhanced the ability of CR3 to bind ligand (figure 10a). IMF-1 highly purified by $C_{18}$ reverse phase chromatography (see figure 15), is equally capable of enhancing binding of EC3bi to CR3 (table III) but lipids extracted from unstimulated PMN, which lack IMF-1, could not increase the binding activity of isolated CR3 (figure 10b, "ctrl lipids"). The IMF-1-induced enhancement of EC3bi binding was dependent on the concentration of adsorbed CR3 (figure 10a) as well as on the concentration of IMF-1 added (figure 11). The ability of IMF-1 to enhance binding of purified CR3 to C3bi was not transient because preincubation of adsorbed CR3 for up to 60 minutes with IMF-1 resulted in sustained high binding activity (figure 12). Several additional control experiments indicate that IMF-1 acts by altering the avidity of CR3. The IMF-1 molecule itself did not bind to EC3bi since surfaces treated with IMF-1 alone did not bind EC3bi (figure 10a), and preincubation of EC3bi with IMF-1 did not alter the extent of binding of erythrocytes to receptor-bearing surfaces (table IV). Furthermore, binding of EC3bi to purified CR3 could be blocked by mAb directed against CR3 (figure 10b). Thus, IMF-1 altered the binding activity of purified CR3 in the absence of other cellular factors and therefore might directly affect the receptor. Since IMF-1 cannot change the number of receptors present and is unlikely to change the two dimensional distribution of the adsorbed CR3, IMF-1 may affect CR3 through an allosteric mechanism.

PMN could regulate CR3 binding to ligand by altering the relative abundance of IMF-1. The data has borne out the predictions: 1) exogenous or

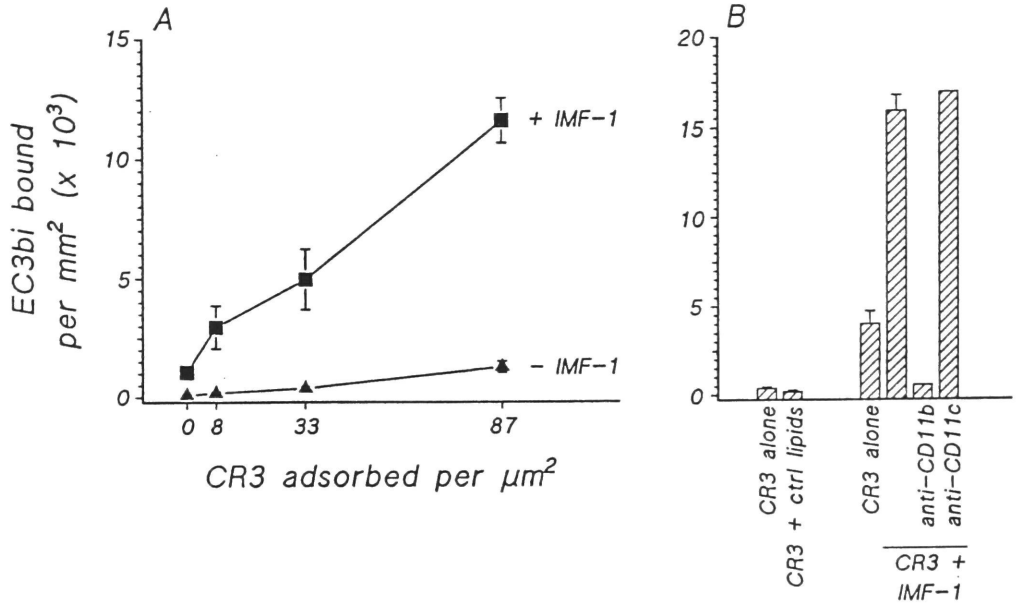



**Figure 10. IMF-1 interacts directly with CR3 to increase binding to ligand.** Panel A. CR3-coated wells were incubated with IMF-1 (165 U/ml) and the binding of EC3bi to the coated surface was determined. Data is the average of 3 experiments, performed in triplicate, bars show the SEM.
Panel B. Wells were coated with 33 CR3 molecules per $\mu m^2$ (two bars at left) or 87 CR3 per $\mu m^2$ (four bars at right), incubated with IMF-1 or lipids extracted from cells lacking IMF-1 ("ctrl lipids"), and washed. IMF-1-induced binding of EC3bi to receptor was also measured in the presence of an anti-CD11b antibody, 44a, or an anti-CD11c antibody, LeuM5, as a control. Both antibodies were used at 50 μg/ml. Data is the average of 3 experiments, performed in triplicate, bars show the SEM.

*PURIFIED IMF-1 INCREASES BINDING OF EC3bi TO PURIFIED CR3*

| | CR3 preincubation | EC3bi bound per $mm^2$ |
|---|---|---|
| CR3 ($87/\mu m^2$) | alone | 280± 56 |
| | + control | 240± 56 |
| | + purified IMF-1 | 1800±400 |
| no CR3 | + purified IMF-1 | 160±112 |

**Table III.** 70,000 units of IMF-1 were fractionated by reverse phase chromatography (see chapter 4). A 15 µl aliquot of a 2.5 ml fraction containing IMF-1 activity was dried down and resuspended to 20 µl in PBS. This purified IMF-1 was applied to CR3-coated culture wells for 30 minutes at room temperature. A similar aliquot from an immediately adjacent fraction, lacking IMF-1 activity in the rosetting assay, was used as a control.

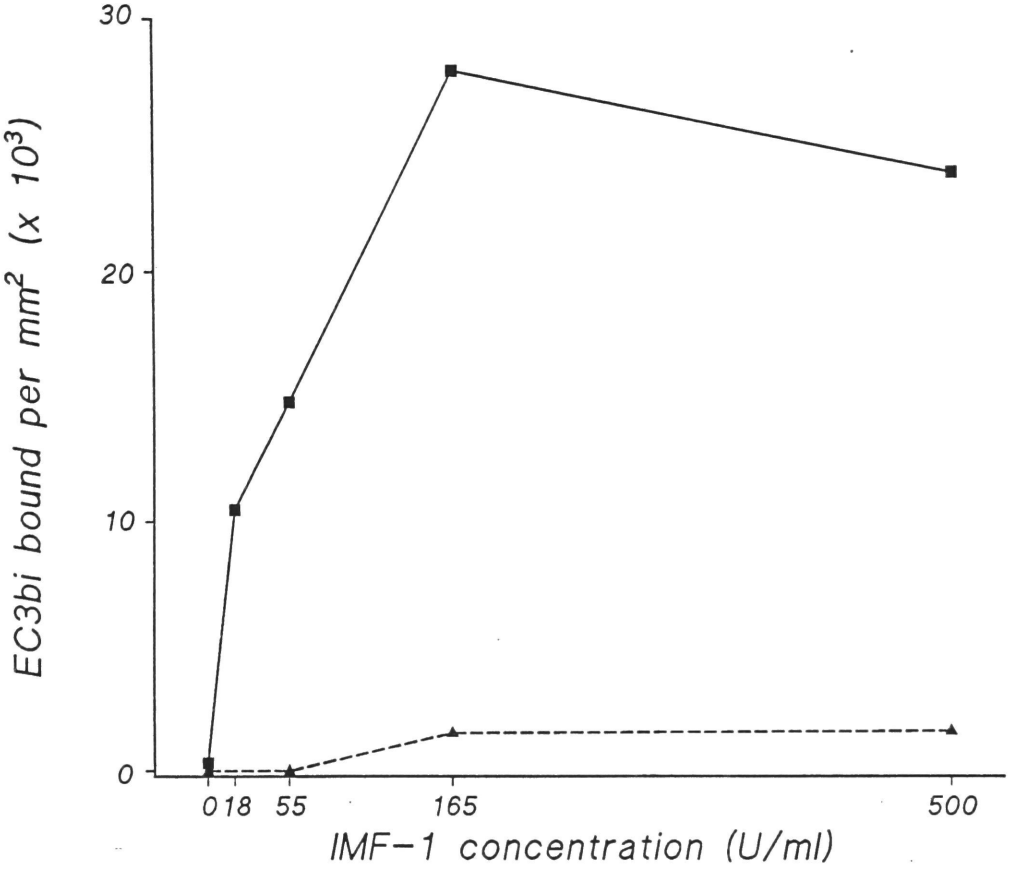



**Figure 11. IMF-1 increases binding of purified CR3 to EC3bi in a dose-dependent manner.**

CR3-coated culture wells were incubated with varying amounts of partially purified IMF-1 (isolated from fNLLP treated cells) in the purified CR3 assay (squares). Uncoated wells incubated with IMF-1 were unable to mediate binding to EC3bi (triangles).

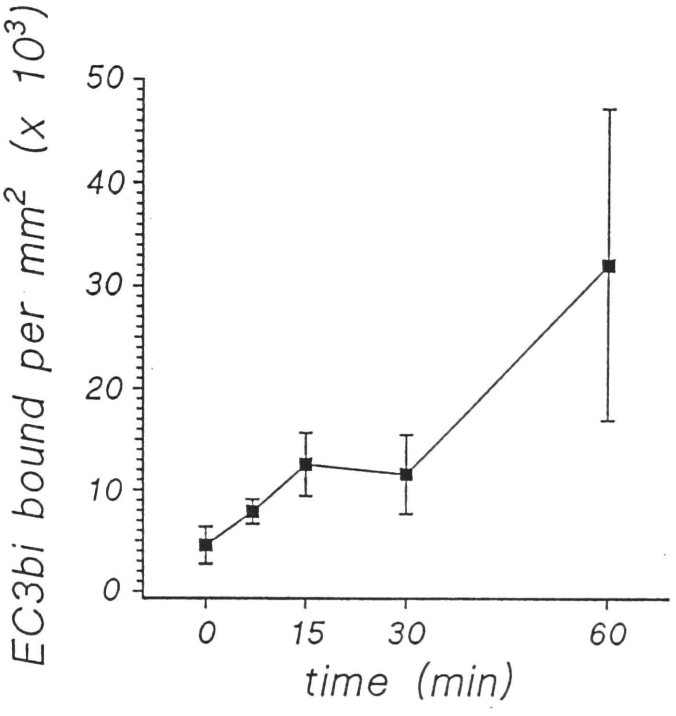



**Figure 12. The effect of IMF-1 on purified CR3 is time-dependent.**
CR3-coated wells (87 CR3/$\mu m^2$) were incubated with IMF-1 (165 U/ml) for varying times before binding to EC3bi was measured.

*PREINCUBATION OF EC3bi WITH IMF-1 DOES NOT INCREASE BINDING TO CR3*

| | EC3bi preincubation | EC3bi bound per $mm^2$ |
|---|---|---|
| CR3 ($87/\mu m^2$) | alone | 5760±2320 |
| | + IMF-1, 55 U/ml | 4160±1680 |
| | + IMF-1, 165 U/ml | 2640±1280 |
| CR3 ($33/\mu m^2$) | alone | 1260± 200 |
| | + IMF-1, 55 U/ml | 960± 264 |
| | + IMF-1, 165 U/ml | 1000± 272 |

**Table IV.** EC3bi ($10^9$/ml) were incubated with IMF-1 (isolated from fNLLP treated cells) for 20 minutes at room temperature, washed extensively in $DGVB^{++}$ and resuspended to $10^8$/ml. EC3bi were then allowed to bind to CR3-coated culture wells for 20 minutes at room temperature.

endogenous IMF-1 can increase CR3 binding to ligand, 2) IMF-1 can be extracted from PMN only after stimulation with agonists with a time course that matches the transient enhancement of CR3 binding, and 3) IMF-1 regulates CR3 function by interacting directly with the receptor.

The remaining chapters will present the current state of knowledge of the chemical properties of IMF-1 and will present more data on the biology of IMF-1 function.

# *Chapter 4 - The chemistry of IMF-1*

**Isolation of IMF-1**

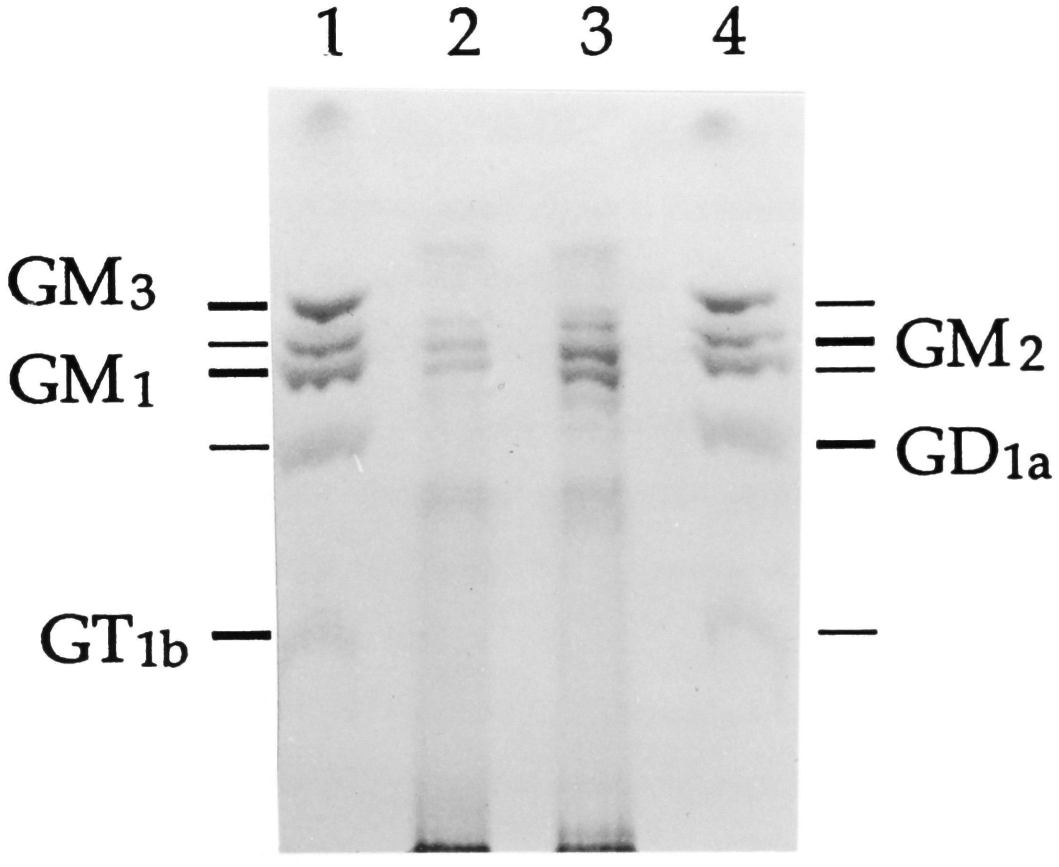
IMF-1 can be extracted from cell pellets using a modified ganglioside purification scheme (see chapter 2). Briefly, lipids are extracted using chloroform:methanol:water=10:10:1, and acidic lipids are purified from the extract by anion exchange chromatography on DEAE sephadex, followed by desalting with a $C_{18}$ Sep Pak cartridge. For further purification, IMF-1 can be separated from contaminants by a Folch partitioning step, in which IMF-1 and other acidic lipids localize to the aqueous phase. Less than 0.1% of IMF-1 activity can be found in the flowthrough from the DEAE and Sep Pak columns. Total yield of IMF-1 ranges from 10 to 33% of starting activity. IMF-1 partitions to the butanol phase in a butanol:$H_2O$=1:1 two phase system. Based on solubility and chromatographic properties, IMF-1 is an acidic, amphiphilic molecule. The preparation of lipids eluted from the Sep Pak column will be referred to as the partially purified lipid extract, and this material was used in the preceding biological characterization experiments on IMF-1, unless otherwise specified.

**IMF-1 is a single, small molecular species.**

The partially purified lipid extract contains several acidic lipids, visible as a number of bands by thin layer chromatography (figure 13). To determine if IMF-1 is a single species, the lipids were further fractionated on a $C_{18}$ reverse

**Figure 13. Thin layer chromatography of partially purified lipid extract.**

25 units of IMF-1 in a partially purified lipid extract from PMN treated with PMA (lane 2) or extract from an equivalent number of unstimulated PMN (lane 3) were spotted onto a high performance thin layer chromatography silica gel 60 plate (E. Merck). Ganglioside controls were run in lanes 1 and 4. The plate was developed in chloroform:methanol:0.2% $CaCl_2$ (aq)=55:45:10. The plate was stained with iodine vapor and photographed immediately.


1
2
3
4
GM3
GM1
GT1b
GM2
GD1a

phase column (figures 14 and 15). IMF-1 eluted as a single peak of activity at approximately 50% acetonitrile, that did not correspond with any peak of absorbance.

**Ultraviolet and visible spectroscopy.**

An ultraviolet and visible spectrum of IMF-1 purified on the $C_{18}$ reverse phase column is shown in figure 16, left panel. The solvent blank is shown at right. IMF-1 has a $\lambda_{max}$ at 196 nm with a slight shoulder out to 300 nm. The spectrum was flat between 300 and 800 nm. This result suggests that IMF-1 does not contain peptide bonds, which absorb at 214 nm, nucleic acids, which absorb at 260 nm, or conjugated double bond systems, which absorb at wavelengths over 300 nm. Since the amount and extinction coefficient of IMF-1 is unknown, it is possible that there was insufficient material present to produce a characteristic absorbance peak. The very large amount of bioactivity present in the sample and the appearance of a peak at 196 nm argues against this interpretation.

The fine discrimination achievable by reverse phase chromatography, coupled with the apparent purity of material suggested by the simple and uncluttered UV spectrum, argue for a high degree of purity of the IMF-1 prepared by reverse phase chromatography.

**None of a wide spectrum of lipids have IMF-1 activity.**

In an effort to identify IMF-1, many well characterized lipids were tested for IMF-1 activity in the rosetting assay. Table V lists those tested, none of which were able to enhance rosetting of PMN to EC3bi.

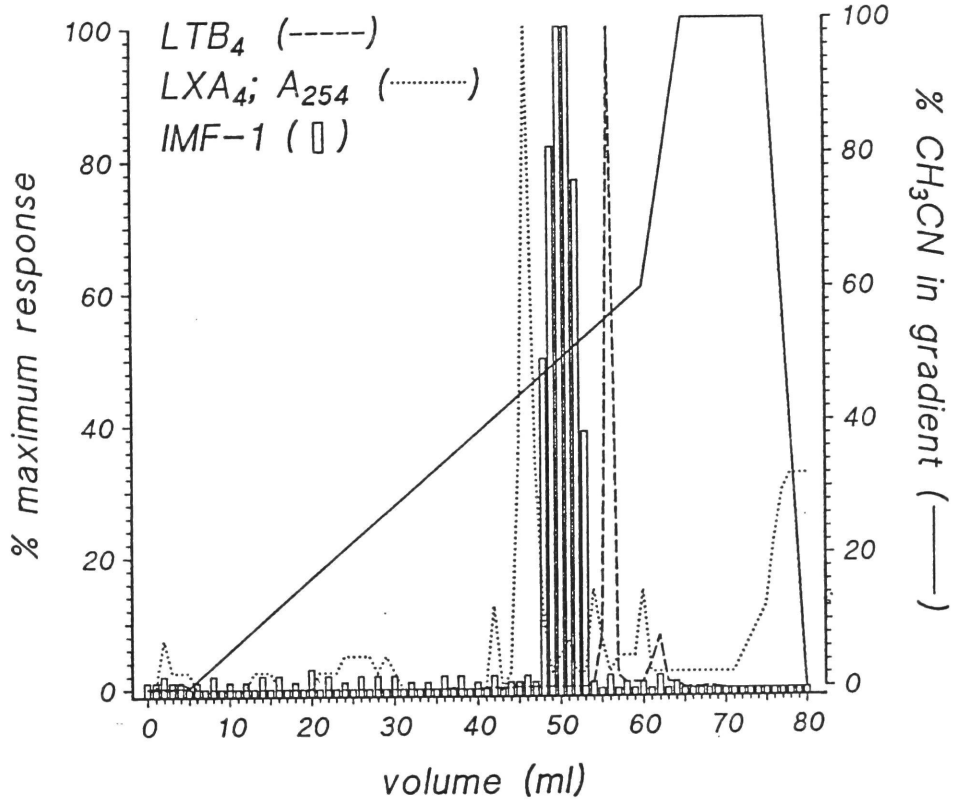



**Figure 14. $C_{18}$ reverse phase chromatography of IMF-1, $LTB_4$ and $LXA_4$.** A Waters μBondaPak $C_{18}$ reverse phase column (10 μm particle size, 3.9mm x 150mm) was loaded with IMF-1 (50 units, partially purified from fNLLP treated cells), $LTB_4$ (5 ng, and 0.5μCi) or $LXA_4$ (5 μg) and eluted with a 1% per minute gradient of water to acetonitrile (solid line). The dotted line corresponds to the chromatogram for the $LXA_4$ run, $A_{254}$. IMF-1 elution was monitored by rosetting assay (histogram), $LTB_4$ by cpm per fraction (dashed line) and $LXA_4$ by absorbance at 254 nm (dotted line).

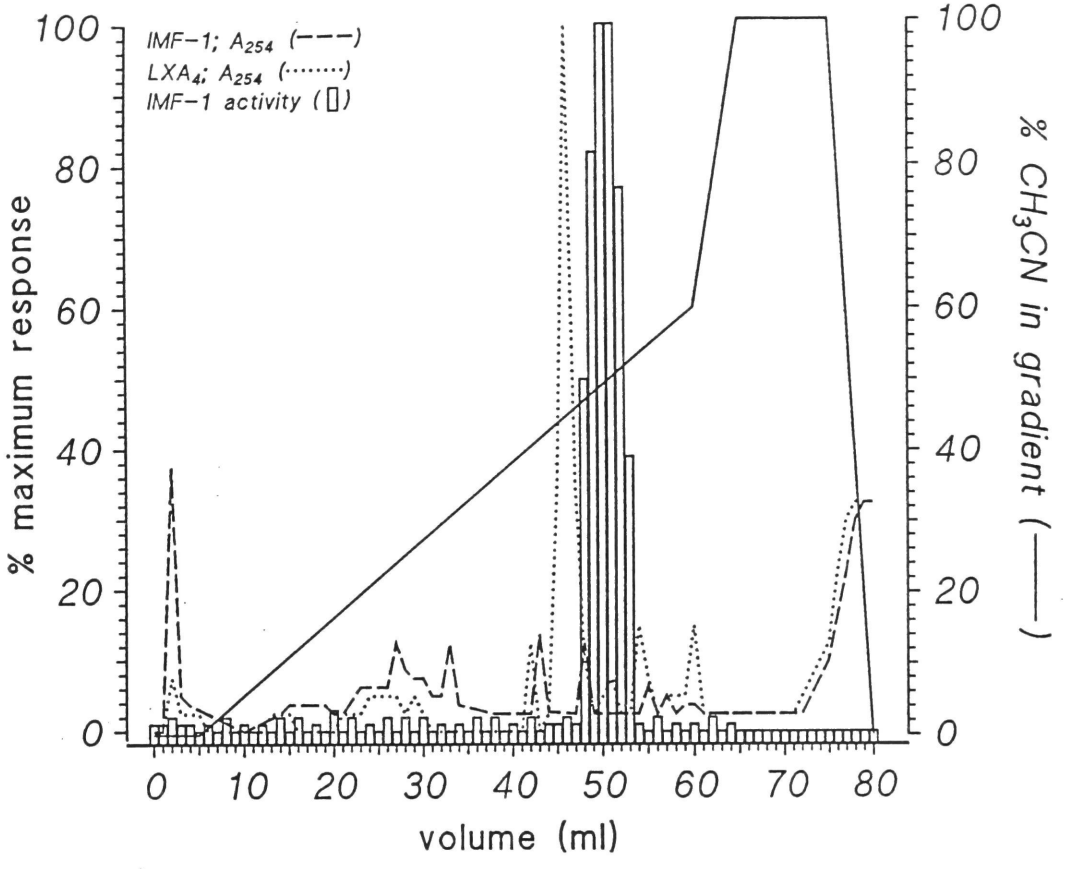



**Figure 15. $C_{18}$ reverse phase chromatography of IMF-1 and $LXA_4$.**
A Waters µBondaPak $C_{18}$ reverse phase column (10 µm particle size, 3.9mm x 150mm) was loaded with IMF-1 (50 units, partially purified from fNLLP treated cells) or $LXA_4$ (5 µg) and eluted with a 1% per minute gradient of water to acetonitrile (solid line). The dotted line corresponds to the chromatogram for the $LXA_4$ run, $A_{254}$. The dashed line represents the chromatogram for the IMF-1 run, $A_{254}$. IMF-1 activity was monitored by rosetting assay (histogram).

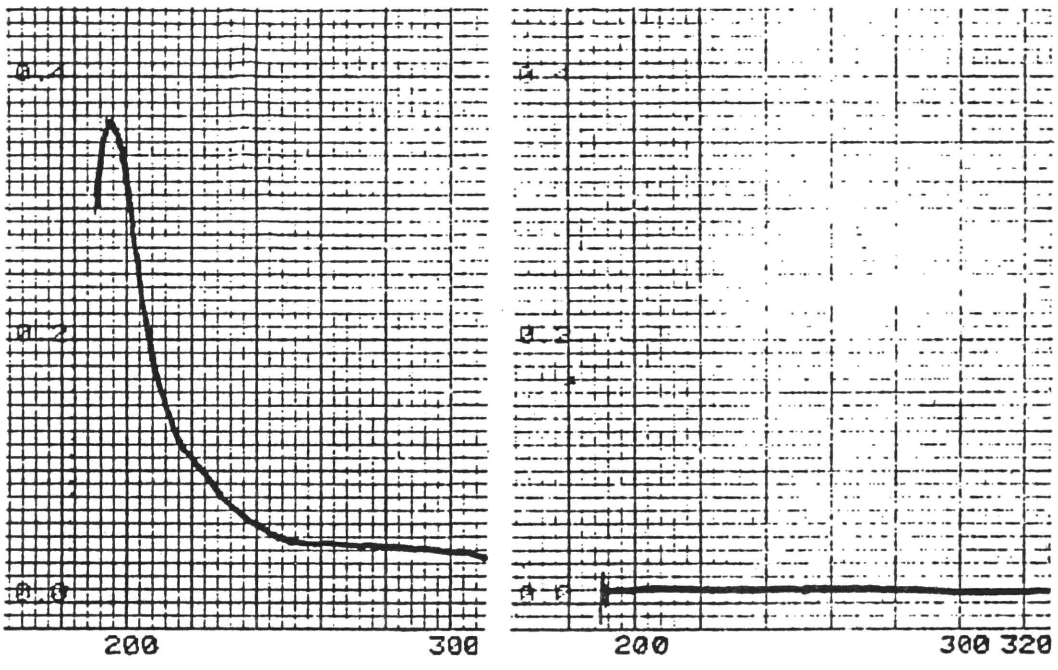



**Figure 16. Ultraviolet and visible spectrum of purified IMF-1.**
A Perkin-Elmer Lambda 5 UV/Vis Spectrophotometer, equipped with a double beam and matched quartz cuvettes, was zeroed with acetonitrile in both cells (at right) and then IMF-1 purified by reverse phase chromatography (52,000 u/ml in acetonitrile) was added to one cell and a spectrum obtained (at left).

*NONE OF THE FOLLOWING HAVE IMF-1 ACTIVITY IN THE ROSETTING ASSAY.*

**at 100 µg/ml:**

GM1, asialo GM1
GM2, asialo GM2
GM3
GD1a, GD1b
GD2, GD3
GT1b
lactosylceramide
globoside
sulfatide
bovine brain gangliosides

**at 15, 1.5, 0.15 µg/ml:**

geranyl pyrophosphate

**at 50, 5 and 0.5 µg/ml:**

GQ1b
PC, dimyristoyl PC
myristic anhydride
phosphatidic acid: dilauroyl, dimyristoyl, dioleoyl
lysophosphatidic acid
myoinositol bis phosphate
lysophosphatidylinositol
dimyristoyl phosphatidyl glycerol
dolichol monophosphate
dimethyl sphingosine

**at 3, 1.5, 0.3 µg/ml:**

sphingosine

**at 0.3, 0.03, 0.003 µg/ml:**

9-hydroxy linoleic acid
13-hydroxy linoleic acid

**at 50, 5, 0.5 µM:**

farnesol
farnesyl pyrophosphate
cis geranylgeraniol
cis geranylgeranyl pyrophosphate
trans geranylgeraniol
trans geranylgeranyl pyrophosphate
solanisol
solanisyl phosphate
solanisyl pyrophosphate

**Table V.** The lipids listed were dissolved in PBS, sonicated, and incubated with PMN for 15 minutes in the rosetting assay.

It is possible that the PMA used to activate the extracted PMN may be a contaminant in the partially purified lipid extract and is responsible for IMF-1 activity. Several pieces of evidence indicate that this is not the case. PMA is not anionic and appears in the flow through of the anion exchange column, rather than cofractionating with IMF-1 in the eluate. PMA is sensitive to hydrolysis in water and the extract is in aqueous solution for a minimum of 12 hours during the purification. Purification of IMF-1 from cells treated with [$^3$H]-PMA yields only background levels of radioactivity in the IMF-1 fractions. These represent at most 0.03 ng/ml of potential contaminating PMA. This concentration is three orders of magnitude less than normally added to cells and 100 times less than the minimum needed to activate phagocytosis in macrophages (Wright and Silverstein, 1982). Finally, and most convincingly, treatment of PMN with a variety of agonists other than PMA, both lipids and proteins, all result in IMF-1 production. IMF-1 is produced by PMN stimulated with tumor necrosis factor (TNF, 5 x $10^3$ u/ml, 15′), platelet activating factor (PAF, 100 nM, 15′) and formyl-norLeu-Leu-Phe (fNLLP, 5 x $10^{-8}$M, 5-15′) as well as by PMN stimulated with PMA (30 ng/ml, 20′). The native conformation of proteins can be completely disrupted by exposure to organic solvents. Tumor necrosis factor, for example, would be unable to maintain its active configuration after extraction in chloroform/methanol, and so is unlikely to be a contaminant responsible for the CR3 modulating activity in the extract.

Platelet activating factor (PAF) is another potential contaminant in the lipid extract that may account for IMF-1 activity. PAF, or L-α-phosphatidylcholine, β-acetyl-γ-O-alkyl, is a lipid produced by PMN in response to agonists (Baggiolini et al.,1988), that can increase CR3 binding activity. To rule out that IMF-1 is equivalent to PAF, a PAF receptor antagonist, WEB 2086 (Casals-Stenzel et al.,1987), was added at concentrations that completely block 100 nM PAF-induced rosetting of EC3bi. The antagonist had no effect on IMF-1-induced rosetting (table VI).

LPS is yet another potential contaminant of the IMF-1 preparation, since it is often found contaminating laboratory solutions and glassware. Unlike LPS, IMF-1 (42 units/ml) does not cause TNF production in whole blood, suggesting IMF-1 activity is not due to contaminating LPS and suggesting that IMF-1 does not function similarly to LPS (figure 17).

**IMF-1 is resistant to a variety of chemical modifications.**

Classes of compounds with physical properties similar to those of IMF-1 include glycerol- or sphingosine-based lipids, eicosanoids, other fatty acids, isoprenoid lipids and proteins or peptides with a hydrophobic tail. All but isoprenoids were ruled out by subjecting IMF-1 to treatments that destroy particular classes.

In all cases, phosphatidylcholine (5 μg), $GM_1$ (5 μg) or some other lipid containing a susceptible linkage was cleaved in parallel with IMF-1. Quantitative destruction of this control compound was verified by thin layer

chromatography comparison with a mock treated control. The details of the treatments are outlined in table VII and summarized below and in figure 18.

IMF-1 retained activity when treated with base (30% ammonium hydroxide or 1N NaOH) or acetic anhydride, suggesting it does not contain ester-linked fatty acids or a phosphate base. IMF-1 was not affected by desialylation with acetic acid or by neuraminidase, suggesting IMF-1 activity does not reside in a ganglioside. Acid hydrolysis (2N or 0.5N methanolic HCl) did not destroy activity, so IMF-1 does not have a sphingosine or glycerol backbone, or glycosidic bonds that are needed for activity. IMF-1 activity is unaffected by phosphatase, hydrofluoric acid or protease, suggesting that phosphate groups are not essential for activity and and that IMF-1 does not have a protein structure. In addition, reduction with sodium borohydride, oxidation with hydrogen peroxide, and periodate oxidation of vicinal hydroxyls all had no effect on IMF-1 activity. Resistance to nitrous acid deamination suggests IMF-1 does not contain primary amines. Of the original categories of candidate structures for IMF-1, the only ones that would remain unaffected by these treatments are isoprenoids, prostaglandins and fatty acids. A protease-resistant peptide with a lipid tail is not a good candidate because IMF-1 does not absorb at 214 nm, the wavelength of absorption of the peptide bond. Prostaglandins and conventional fatty acids are unlikely considering IMF-1 activity partitions to the aqueous phase rather than the organic in a Folch partition, and since prostaglandins contain conjugated double bond systems, ruled out by the spectrograph.

*IMF-1 ACTIVITY IS NOT DUE TO EXTRACTED PLATELET ACTIVATING FACTOR.*

| agonist | inhibitor | EC3bi (AI) |
|---|---|---|
| none | none | 112 |
| PAF, 100 nM | none | 370 |
| PAF, 100 nM | WEB 2086, 10μM | 106 |
| PAF, 1 nM | none | 312 |
| PAF, 1 nM | WEB 2086, 10μM | 169 |
| IMF-1, 42 u/ml | none | 652 |
| IMF-1, 42 u/ml | WEB 2086, 10μM | 575 |

**Table VI.** PMN were treated with PAF or IMF-1 for 15′ in the rosetting assay in the presence or absence of the PAF antagonist, WEB 2086. Cells were washed and allowed to interact with EC3bi.

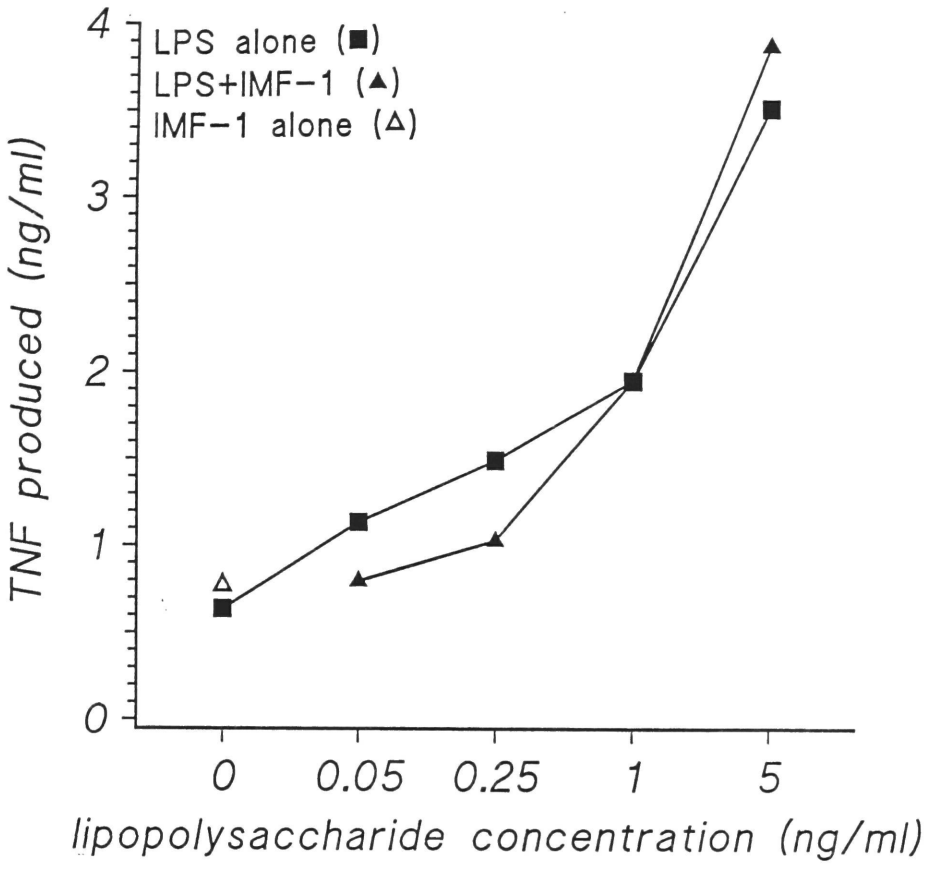



**Figure 17. IMF-1 does not contain or act like LPS.**
Whole blood was incubated for 5 hours at 37°C with lipopolysaccharide (LPS), IMF-1 (42 units/ml) or both. After spinning out the cells, supernatants were tested for the presence of tumor necrosis factor (TNF) by radioimmunoassay (Vlassara, et al.,, 1988). This experiment is representative of two repeats.

*IMF-1 ACTIVITY IS UNAFFECTED BY A VARIETY OF CHEMICAL AND ENZYMATIC TREATMENTS.*

- 30% $NH_3OH$:$CH_3OH$ = 1:1, room temperature, overnight
- 1 N NaOH, 75°C, 3 hours
- acetic anhydride:glacial acetic acid=2:3, 150°C, 48 hours
- 1 N acetic acid, 100°C, 45'
- V. cholera neuraminidase, 0.1 U/ml, 37°C, overnight
- 2 N methanolic HCl, 75°C, 5 hours
- 0.5 N methanolic HCl, 100°C, 2 hours
- 50% HF, 48 hours, 0°C
- bacterial alkaline phosphatase, 0.04 U/ml, 37°C, overnight
- proteinase K, 1.6 mU/ml, 37°C, overnight
- 0.5 M $NaBH_4$ in 3 M NaOH, room temperature, overnight
- 2 mM $NaIO_4$ in 70% ethanol, room temperature, overnight
- 30% $H_2O_2$ in 10μM $FeSO_4$, room temperature, overnight
- 0.25 M nitrous acid in 0.1 M acetate buffer, pH 3.8, 8hrs, room temperature

**Table VII.** IMF-1 (10-50 units) was treated as listed or mock treated by leaving out the destructive agent as a control. Reactions were stopped by separating IMF-1 from the destructive agent by evaporation or by resuspension of IMF-1 into the butanol phase of a butanol:water=1:1 two phase system. IMF-1 was then dissolved in PBS and tested against the mock-treated control in the rosetting assay. In all cases, activity remained in both the treated and control samples. In addition, phosphatidylcholine (5 μg) or $G_{M1}$ (5 μg) or some other lipid containing a susceptible linkage was cleaved or mock-treated in parallel with IMF-1. Quantitative destruction of this control compound was verified by thin layer chromatography.

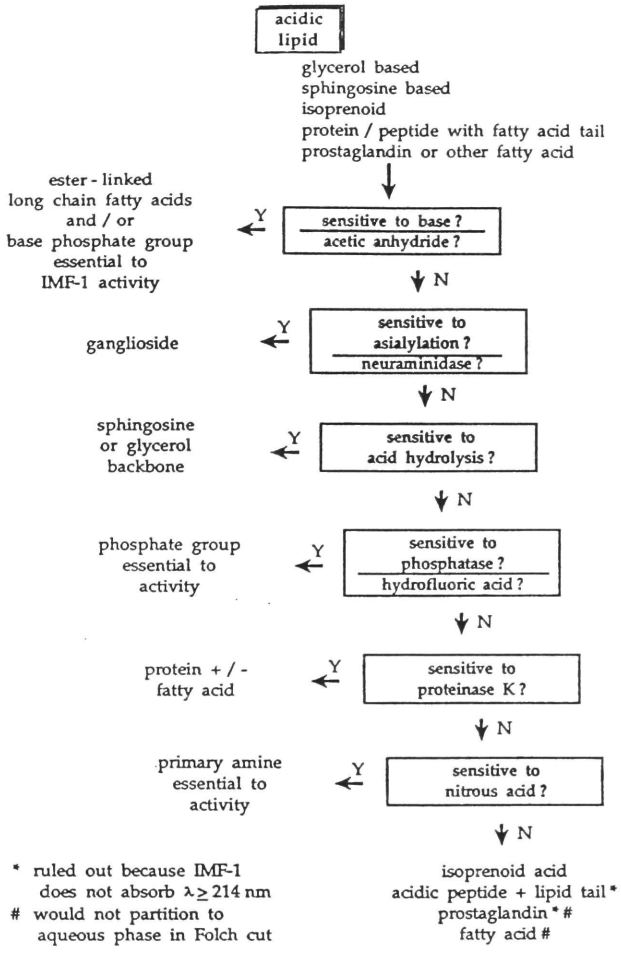



**Figure 18. Flow chart summary of chemical analysis of IMF-1.** See text for discussion.

It may be useful to exploit the properties of IMF-1 outlined above to devise more efficient purification protocols. For example, when IMF-1 is extracted from a neutrophil homogenate treated with 0.16 N sulfuric acid, the resulting chloroform/methanol extract of the cellular material is far less turbid. This result suggests contaminating lipids in the IMF-1 extract may be removed due to their susceptibility to acid or other treatments that do not affect IMF-1. However, the IMF-1 produced by such a method would have to be compared with IMF-1 prepared in the standard purification protocol to verify that acid or other treatments did not change the nature of IMF-1 without destroying its activity.

One chemical treatment that was found to destroy IMF-1 activity was ozonolysis (table VIII). This treatment adds oxygen across carbon-carbon double bonds and results in double bond cleavage, forming two aldehyde molecules. Ozone is consumed as it reacts with targets, making ozone the limiting factor in these reactions and for this reason destruction of IMF-1 could be observed using $C_{18}$-purified IMF-1 but not partially purified IMF-1. It is unlikely that the treatments listed in table VII were ineffective due to the presence of competing contaminants since, in the non-enzymatic reactions, a large excess of the destructive reagents were added in each case. In all the treatments listed, a parallel reaction was run in which a molecule containing a susceptible structure was quantitatively destroyed, as monitored by TLC. Due to the inability to detect IMF-1 by physical means, IMF-1 was followed

*OZONOLYSIS DESTROYS IMF-1 ACTIVITY*

| stimulus | binding of EC3bi (AI) |
|---|---|
| resting PMN | 139 |
| PMA | 1000 |
| partially purified IMF-1, 58 U/ml | 445 |
| purified IMF-1, 58 U/ml | 500 |
| purified IMF-1, + $O_3$, 175 U/ml | 95 |

**Table VIII.** IMF-1 (partially purified or purified by size exclusion chromatography) in ethyl acetate was treated with 2 ppm $O_3$ for 15′ at 20°C. O3 was generated by passing a stream of oxygen over an ultraviolet light source. Samples were dried down, and residue dissolved in PBS. IMF-1 activity was assessed in the rosetting assay.

by the loss of bioactivity after treatment, leaving open the possibility that IMF-1 was cleaved but the bioactivity was unaffected.

**IMF-1 has a molecular weight of approximately 340 daltons.**

Size exclusion chromatography was used to determine the molecular weight of IMF-1. A TSK G2000HXL size exclusion column with a separation range of up to 2000 daltons was used. An isocratic run in tetrahydrofuran separated molecular weight standards to yield a calibration curve shown in figure 19 by the dark triangles. IMF-1, as measured by the rosette-inducing activity in each fraction, ran at 6.4 and 9.4 ml (histogram). Peaks of IMF-1 activity do not correspond to peaks of absorbance at 254 nm, as expected from the UV spectrum data. The 9.4 ml fraction corresponds to a molecular weight of 340 daltons $\pm$ 16. The 6.4 ml fraction is in the excluded volume of the column, suggesting that the IMF-1 running there is in micelles, since it has a molecular weight >>2000. Rerun of the material eluting at the excluded volume (figure 20) yielded the two molecular weight species of IMF-1 activity. A rerun of the 340 MW fraction, after mixing with phosphatidylcholine (figure 21), also yielded the two forms of IMF-1. It appears that the two IMF-1 activities are in equilibrium with each other because they can interconvert and thus correspond to the same molecule running in either a monomeric (340) or micellar (>>2000) form. The possibility exists that the apparent IMF-1 molecular weight of 340 daltons is inaccurate due to interactions between IMF-1 and the column sorbent or tetrahydrofuran. Fractionation of IMF-1 on a distinct sizing column or on this column but using a different solvent might

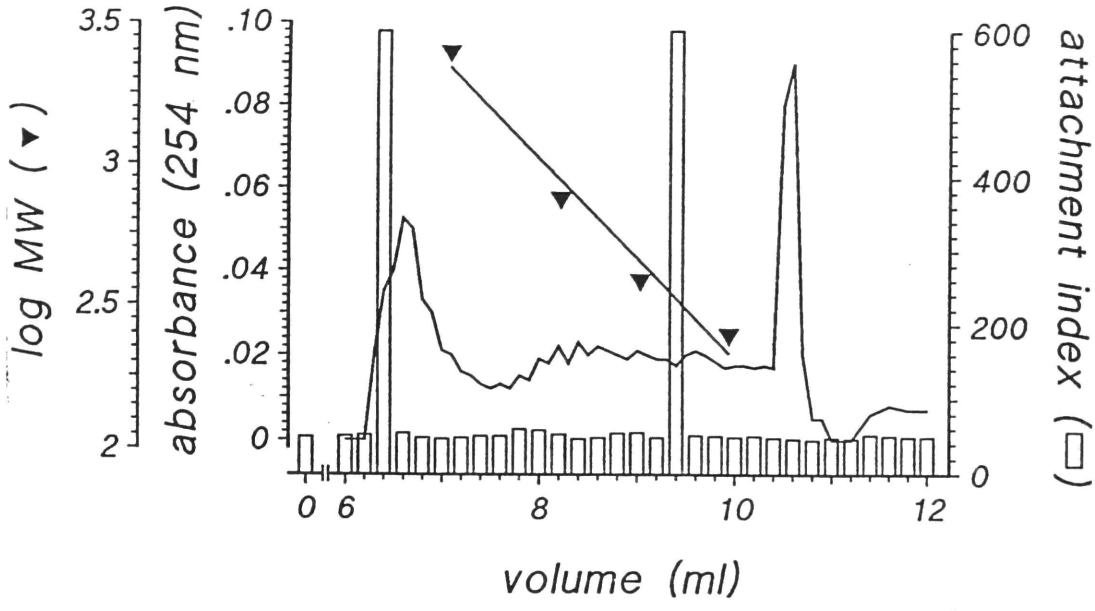



**Figure 19. Size exclusion chromatography on IMF-1.**
IMF-1 was separated on a TSK G2000HXL size exclusion chromatography column run in tetrahydrofuran. Elution volumes of polystyrene molecular weight standards in the range of 1000 - 180 daltons are shown (triangles). Linear regression yields a standard curve of $y = -1.278x + 3.302$ with $r^2=0.9884$. IMF-1 activity (histogram) of each fraction was determined by rosetting assay.

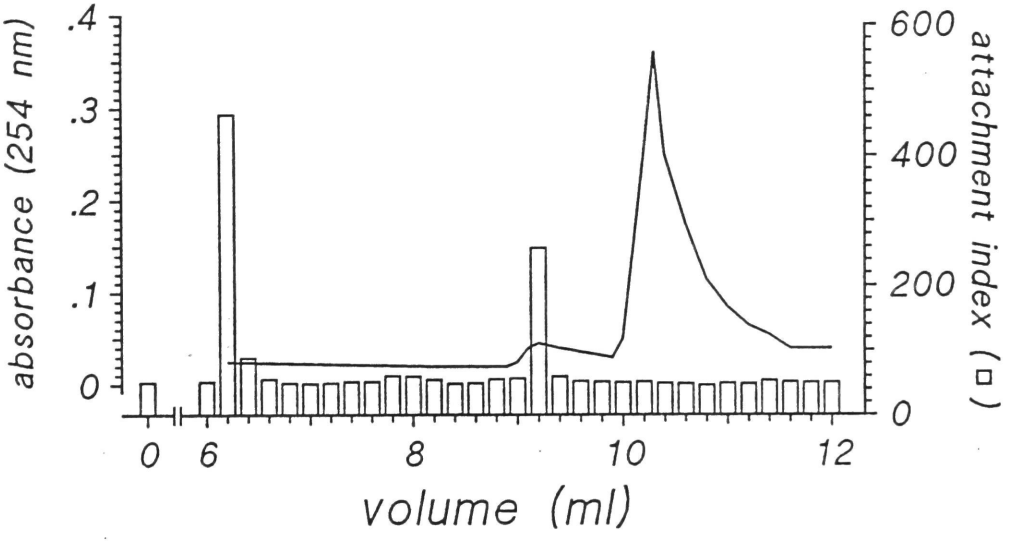



**Figure 20. Sixe exclusion chromatography on the excluded volume IMF-1 activity.**

60 U of partially purified IMF-1 was run on a TSK G2000HXL column as described in figure 19. The fraction in the excluded volume of the column containing IMF-1 activity was dried down and resuspended into tetrahydrofuran. Rechromatography yielded IMF-1 activity eluting at two positions, as determined by rosetting assay on material in each fraction. This experiment is representative of five repeats.

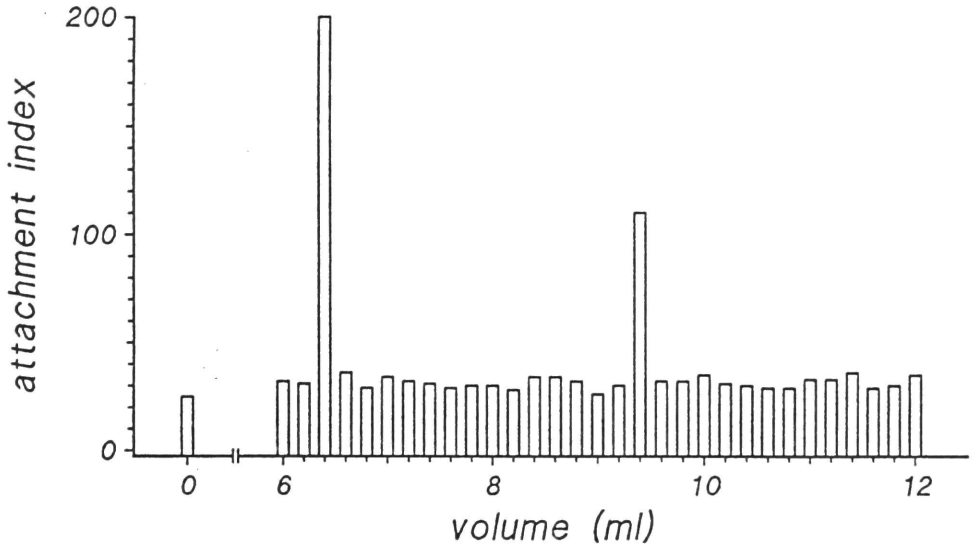



**Figure 21. Sixe exclusion chromatography on the 340 dalton IMF-1 activity.** 330 U of partially purified IMF-1 was run on a TSK G2000HXL column as described in figure 19. The fraction eluting at a position corresponding to a molecular weight of 340 daltons was dried down and resuspended into tetrahydrofuran containing 100 µg phosphatidylcholine (dilinoleoyl). Rechromatography yielded IMF-1 activity eluting at two positions, as determined by rosetting assay on material in each fraction. Absorbance of eluting material was not recorded due to detector malfunction.

help to rule this out.

**IMF-1 is not equivalent to leukotriene B4 or lipoxin A4.**

The apparent molecular weight of of IMF-1 of 340 daltons argues against a ganglioside structure (MW>1000), as well as a glycerophosphatide (MW>500), but it is suggestive of an eicosanoid. To rule out leukotriene $B_4$ ($LTB_4$) and lipoxin $A_4$ ($LXA_4$), two candidate eicosanoids that function as neutrophil agonists (table IX, and SD Wright, unpublished), IMF-1 was runalongside $^3$H-$LTB_4$ and $LXA_4$ on a Waters μBondaPak $C_{18}$ reverse phase column (figures 14 and 15). The gradient was 1% per minute water to acetonitrile run from 0-60% acetonitrile (see solid line on graph for gradient profile). These conditions were known from previous runs on this column to elute IMF-1 at 45-50% acetonitrile. The $LTB_4$ elution position was monitored by cpm present in each fraction (dashed line), $LXA_4$ was monitored by absorbance at 254 nm (dotted line) and IMF-1 position was verified by assaying fractions for activity in the rosetting assay (histogram corresponds to attachment index). Neither eicosanoid coeluted with IMF-1. Moreover, concentrations of $LTB_4$ or $LXA_4$ capable of activating CR3 gave large peaks of absorbance at 254 nm.

**IMF-1 does not appear to be one of the known lipid products of PMN.**

PMN are known to produce a number of lipids in response to cellular activation including arachidonate metabolites and platelet activating factor (PAF) (Henson et al.,1988). Table VI shows that IMF-1 is not identical to PAF, since a PAF receptor antagonist has no effect on IMF-1-induced rosetting.

*Lipoxin $A_4$ increases CR3 binding to C3bi-coated erythrocytes*

| stimulus | AI |
|---|---|
| PMN alone | 56 |
| lipoxin $A_4$ | |
| 50 µg/ml | 313 |
| 5 µg/ml | 288 |
| 500 ng /ml | 109 |
| 50 ng/ml | 48 |

**Table IX.** Various concentrations of lipoxin $A_4$ were applied to PMN for 15 minutes and binding to EC3bi was measured in the rosetting assay.

A major class of bioactive lipids produced by PMN are derived from arachidonic acid. Arachidonate is liberated from the 2 position of glycerol in membrane phospholipids by the action of phospholipase A2 (Roberts et al.,1977). It is then metabolized by cyclooxygenase to prostaglandins and by lipoxygenases to leukotrienes, lipoxins and other hydroxylated eicosanoids. PMN were incubated with inhibitors of 5-lipoxygenase (MK886 (Rouzer et al.,1990) and L-651,392 (Guindon et al.,1987), kindly provided by Dr. S. Longpre, Merck), cyclooxygenase (indomethacin), and phospholipases $A_2$ and C (bromophenacyl bromide) (Roberts et al.,1977) and in all cases, the inhibitor alone or in combination with PMA or IMF-1 did not change the extent of CR3 binding to EC3bi over that seen in parallel wells which were not exposed to the inhibitor (table X). These data suggest IMF-1 is not PAF or a leukotriene, lipoxin, prostaglandin or other arachidonate metabolite. To verify that these inhibitors did block release of arachidonate and subsequent metabolism, PMN will be loaded with radioactive arachidonate and metabolites will be measured in control and drug-treated cells. These experiments are ongoing.

**Production of IMF-1 requires a mevalonate precursor.**

To address the possibility that IMF-1 is an isoprenoid, an attempt was made to block production of IMF-1 in response to agonists by blocking the rate limiting enzyme in isoprenoid synthesis, HMGCoA reductase (figure 22). A competitive inhibitor of the enzyme, lovastatin (40 μg/ml), was added to the cells to block enzyme activity. Then, to deplete the cells of any IMF-1 precursors that had passed this early step in the synthesis pathway,

*INHIBITORS OF PRODUCTION OF ARACHIDONATE METABOLITES DO NOT AFFECT CR3 ACTIVATION*

| inhibitor | | attachment index |
|---|---|---|
| MK886, 50 nM | | 37 |
| | + IMF-1 | 423 |
| | + PMA | 431 |
| L651,392, 5 μM | | 51 |
| | + IMF-1 | 406 |
| | + PMA | 424 |
| none | | 52 |
| | + IMF-1 | 417 |
| | + PMA | 400 |
| indomethacin, 10 μM | | 20 |
| | + IMF-1 | 160 |
| | + PMA | 403 |
| none | | 26 |
| | + IMF-1 | 209 |
| | + PMA | 358 |
| bromophenacylbromide, 3 μM | | 31 |
| | + IMF-1 | 336 |
| | + PMA | 425 |
| none | | 23 |
| | + IMF-1 | 340 |
| | + PMA | 471 |

**Table X.** PMN were treated with indomethacin (100′), MK886 (50′), L651,392 (50′) or bromphenacylbromide (70′) in the rosetting assay, in the presence or absence of IMF-1 (42 U/ml) or PMA (30 ng/ml). EC3bi were allowed to adhere to cells for the last 15 minutes of incubation. These experiments are representative of at least two repeats.

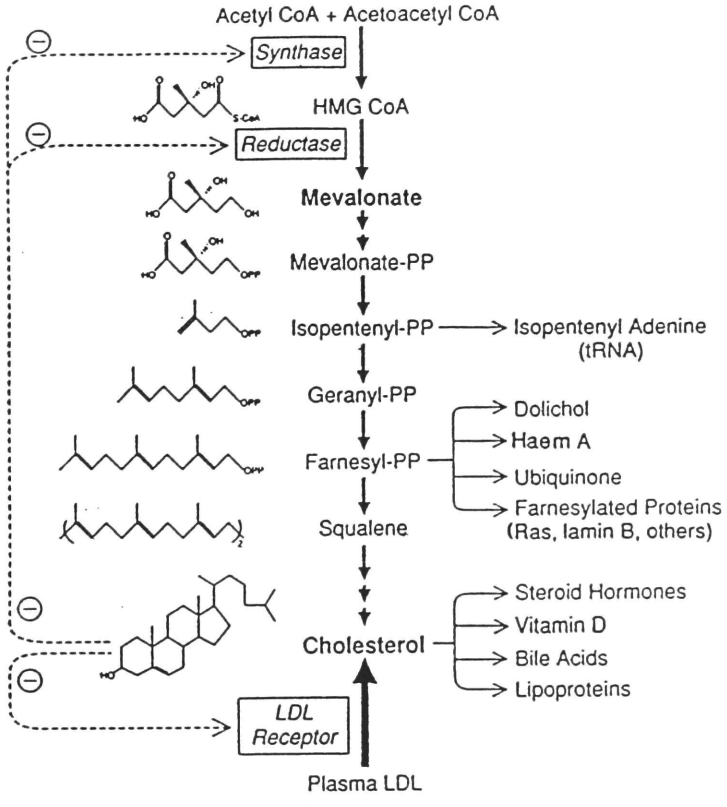



**Figure 22. The mevalonate pathway in animal cells.** (from Goldstein and Brown, 1990)

C5a ($10^{-8}$M) was added. C5a transiently activates cells without leaving them refractory to subsequent stimulation by PMA (Lo et al.,1989). Lovastatin-treated cells, briefly pulsed with C5a, were then activated with PMA to see if PMA could cause production of IMF-1 despite the block in isoprenoid production. PMA could not increase rosetting in the lovastatin-blocked cells, but could when the block was overcome by adding mevalonate, the product of HMGCoA reductase, to the media (table XI). This experiment supports thepossibility that IMF-1 is a downstream product of the mevalonate synthesis pathway or that the generation of IMF-1 requires a mevalonate metabolite.

In order to differentiate between these two possibilities, PMN were labeled with [$^3$H]-mevalonate and then extracted for IMF-1. It is known from metabolic labeling with 2-[$^{14}$C]-acetate or [$^{14}$C]-mevalonate that PMN can synthesize squalene but not sterols (Fogelman et al.,1977). Cells were incubated with [$^3$H]-mevalonate for 2 hours in the presence of lovastatin, to ensure uptake of exogenous mevalonate. Early in the incubation, IMF-1 precursor was cleared by adding C5a. For the final 15′ of incubation, cells were activated with TNF, in order to stimulate IMF-1 production, before being extracted with organic solvents. 12% of the counts added to the cells were found in the lipid extract derived from the cell pellet. Only background levels of counts coeluted with the IMF-1 in the pellet extract on $C_{18}$ reverse phase chromatography (figure 23). The presence of several peaks of radioactivity in the chromatogram suggest that label was incorporated into mevalonate metabolites. This experiment suggests that IMF-1 itself is not a product of the

*GENERATION OF IMF-1 REQUIRES A PRODUCT OF THE MEVALONATE PATHWAY*

| stimulus | binding of EC3bi (AI) | |
|---|---|---|
| | experiment 1 | experiment 2 |
| resting PMN | 32 | 19 |
| lovastatin | 48 | ND |
| lovastatin + C5a | 86 | 37 |
| C5a + PMA | ND | 309 |
| lovastatin + C5a + PMA | 74 | 50 |
| lovastatin, mevalonate, C5a + PMA | 465 | 281 |
| IMF-1 | 511 | 277 |
| lovastatin, C5a + IMF-1 | 302 | 279 |

**Table XI.** Adherent PMN were incubated in lovastatin (40 µg/ml) for 90 minutes at 37°C in the presence or absence of mevalonate (50 µg/ml). Cells were transiently activated with C5a ($10^{-8}$M) during the incubation and were treated with PMA (30 ng/ml) or IMF-1 (42 U/ml) for the last 20 minutes of the incubation. These two experiments are representative of four repeats.

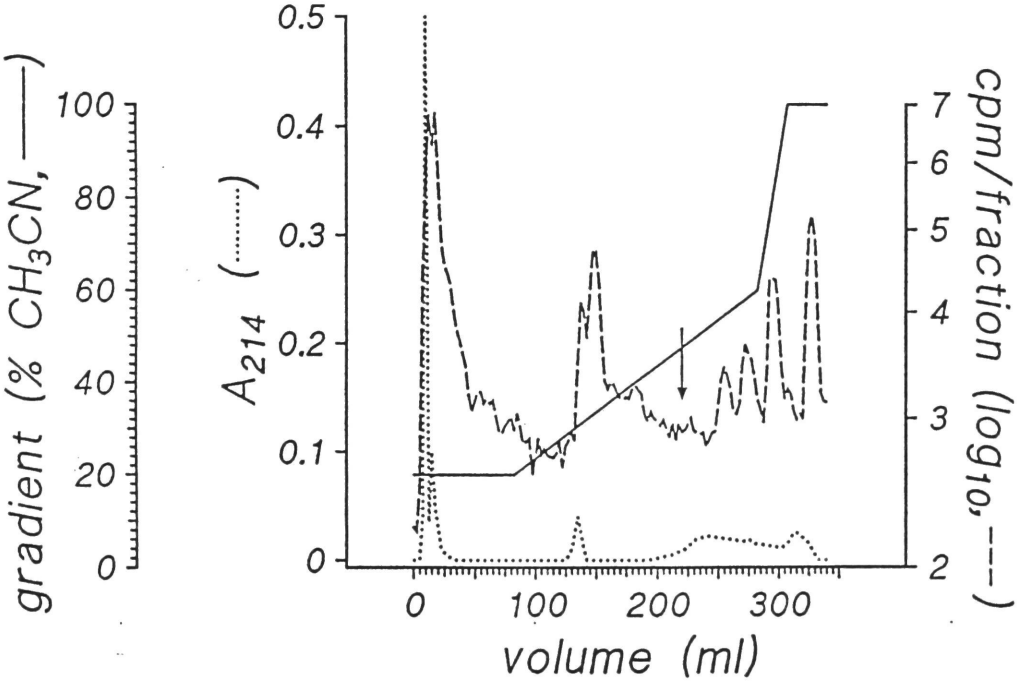



**Figure 23. IMF-1 from [$^3$H]-mevalonate labelled PMN is not radioactive.** PMN ($2 \times 10^8$) were incubated with 500 µCi of [$^3$H]-mevalonate in the presence of lovastatin (40 µg/ml) for 2 hours at 37°C. Cells were transiently activated with C5a during the incubation and were treated with TNF ($5 \times 10^3$ U/ml) for the last 15 minutes of the incubation. Cells were pelleted and extracted with chloroform:methanol:water=10:10:1. About 12% of the counts added were present in this extract. Half of the extract was dried down under $N_2$, resuspended into 20% $CH_3CN$ (aq), and separated by $C_{18}$ reverse phase chromatography. IMF-1 activity eluted at the position indicated by the arrow.

mevalonate pathway, although it is possible that insufficient label was incorporated into the IMF-1 pathway to allow detection.

**GC-MS analysis reveals a candidate peak of 315 daltons**

70,000 units of IMF-1 were purified by $C_{18}$ reverse phase chromatography, derivatized with trimethylsilane (TMS) and loaded onto a capillary gas chromatography column attached to an electron ionization mass spectrometer. Fractions from the reverse phase column containing the IMF-1 activity peak were compared to fractions eluting on either side of the activity peak to rule out common contaminants. Figure 24 shows the IMF-1 GC profile (top) compared to the trailing edge fractions containing slight levels of activity (middle) and leading edge fractions, lacking IMF-1 activity (bottom). A peak (arrowhead) appearing in the sample from fractions with high IMF-1 activity was absent in the sample from fractions with no IMF-1 activity (bottom) and was very small in sample from fractions with trace amounts of IMF-1 activity (middle). A mass spectrum of this peak is shown in figure 25. The top panel shows the TMS derivative while the bottom panel shows the deuterated-TMS derivative. Differences in peak mass between the two spectra show the molecule has two derivatizable groups, which could include hydroxyl, carbonyl or carboxyl oxygens. TMS derivatives give much stronger signals for the mass ion minus a methyl group than they do for the mass ion itself. Given that fact, the molecule has a molecular weight of 315 daltons ($444(M+TMS_2-CH_3) - 144(2 \times TMS) + 15(CH_3)$). The peaks with the highest

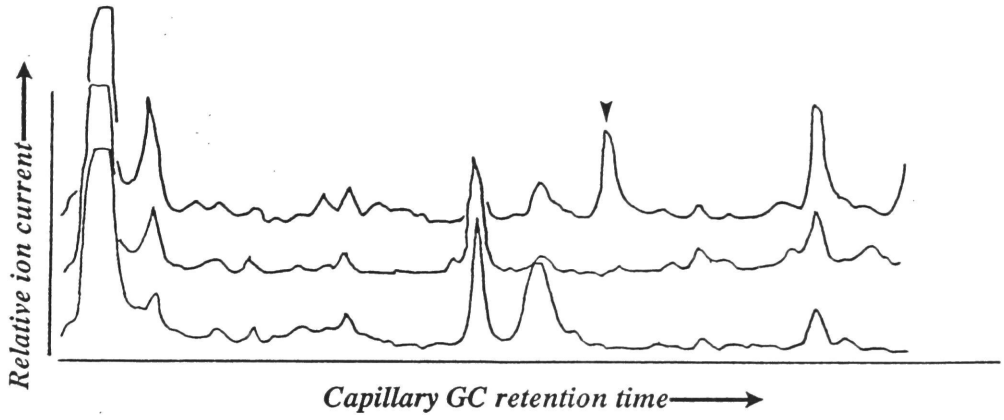



**Figure 24. Gas chromatography of purified IMF-1.**
70,000 units of partially purified IMF-1 was purified on a $C_{18}$ Waters μBondapak reverse phase column (10μm particle size, 3.9mm x 150 mm) in a 1% per minute gradient of 20 - 60% acetonitrile (aq). Elution of IMF-1 at 47% $CH_3CN$ was verified by testing column fractions by rosetting assay. Purified IMF-1 (top tracing), as well as material from fractions eluting just before (bottom tracing) and after (middle tracing) those containing IMF-1 activity, was derivatized with trimethylsilane (or deuterated TMS) using 1:1=BSTFA (Sigma):pyridine, 50°C, 30′ and loaded onto a capillary gas chromatography column in series with an electron ionization mass spectrometer.

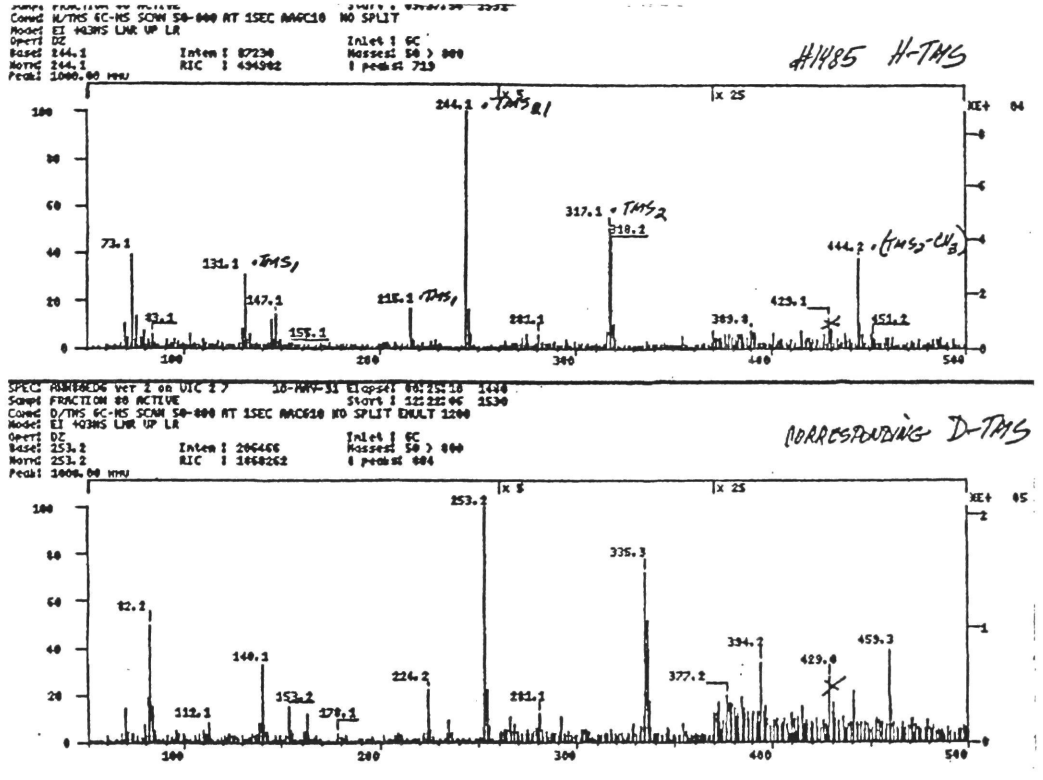



**Figure 25. Mass spectrum of purified IMF-1.**
Spectrum of peak indicated by arrowhead on figure 24. Peak was chosen because it was present in IMF-1-containing fractions but absent in those eluting just before IMF-1 activity, and slight in the fractions following, which contained a low level of IMF-1 activity. The array of fragments shown suggest the peak represents a molecule of underivatized $M_r$ of 315 daltons. See text for discussion.

signals represent two fragments of molecular weights 143 and 172 (317 - 144(2 x TMS) = 173; 244 - 72(1 x TMS) = 172; 215 - 72(1 x TMS) - 142). The 172/173 fragment probably has a carboxyl group, explaining the fact that the fragment can be singly or doubly silylated, and why the molecular weight varies by one, representing the hydroxyl hydrogen. The two predominant fragments of 143 and 172 daltons suggest the presence of a labile bond at roughly the center of the molecule that is directing preferential cleavage at that site. The presence of two predominant fragments also suggests the molecule is not a simple hydrocarbon. The odd molecular weight suggests the presence of a molecule with an odd valency, most likely nitrogen. The absence of a characteristic isoprene fragment of 69 daltons means either the molecule is not an isoprenoid or that the ultimate isoprene repeat(s) on the molecule are saturated, with a molecular weight of 71 daltons, and lack the double bond that directs cleavage of that fragment. The GC-MS analysis will have to be performed on another preparation of IMF-1, preferably containing more material, in order to verify that appearance of this 315 dalton peak correlates with the presence of IMF-1 activity.

In summary, its physical properties suggest IMF-1 is an acidic, amphiphilic lipid. IMF-1 appears to be distinct from the known lipid products of PMN. It has an absorbance maximum at 196 nm and a molecular weight of 340 daltons by size exclusion chromatography. IMF-1 or a molecule involved in its synthesis may be a product of the mevalonate pathway. More studies are required to determine the chemical nature of the molecule.

## *Chapter 5 - Further studies on the biology of IMF-1*

### IMF-1 does not behave like known agonists of PMN

IMF-1 is a molecule that can be extracted from PMN treated with agonist and that can enhance the binding of CR3 to its ligand, C3bi. IMF-1 appears to regulate CR3 function by acting directly on the receptor (figure 10). However, like an agonist, IMF-1 could cause intracellular responses in addition to its effect on CR3. To rule this out IMF-1 was tested for typical agonist-like properties.

Agonist-treated PMN become refractory to restimulation with the same agonist. This property of homologous desensitization is illustrated for PMA in figure 26a, and has also been observed for other agonists such as NAP-1/IL-8 (PA Detmers, unpublished) and C5a (Webster et al.,1980). After treating PMN for 60 minutes with PMA, CR3 activity declined and did not increase upon readdition of PMA (open square, figure 26a). Cells treated for 60 minutes in PMA remained viable and addition of IMF-1 caused increased binding to EC3bi (open triangle, figure 26a). In contrast, cells treated for 60 minutes with IMF-1 exhibited enhanced binding of EC3bi in response to either IMF-1 or PMA (figure 26b), indicating the absence of homologous desensitization to IMF-1.

Agonists of PMN trigger fusion of specific granules (also known as secondary granules) with the plasma membrane. No degranulation occurred in response to doses of IMF-1 as high as 42 U/ml (figure 27). These data

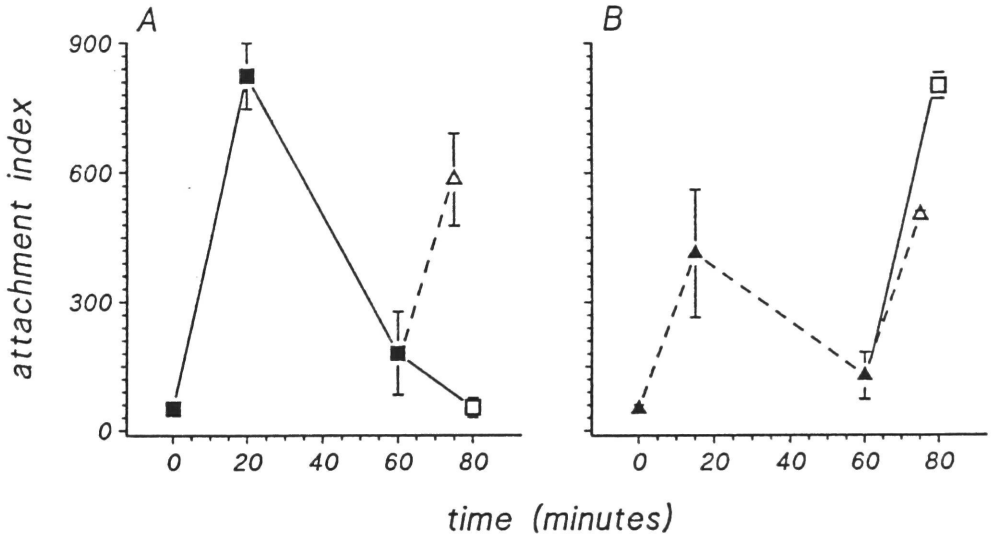



**Figure 26. IMF-1 does not cause homologous or heterologous desensitization.**
PMN were treated for up to 1 hour with PMA (panel A, 30 ng/ml, closed squares, solid lines) or IMF-1 (panel B, 42 U/ml, partially purified from fNLLP treated cells, closed triangles, dashed lines). Binding of EC3bi was measured in the rosetting assay. At 60 minutes, replicate cultures were washed and then treated with either IMF-1 (panels A and B, open triangles, dashed line) or PMA (panels A and B, open squares, solid line) as indicated before attachment index for EC3bi was measured. This data is the average of two experiments, performed in triplicate, bars show the SEM.

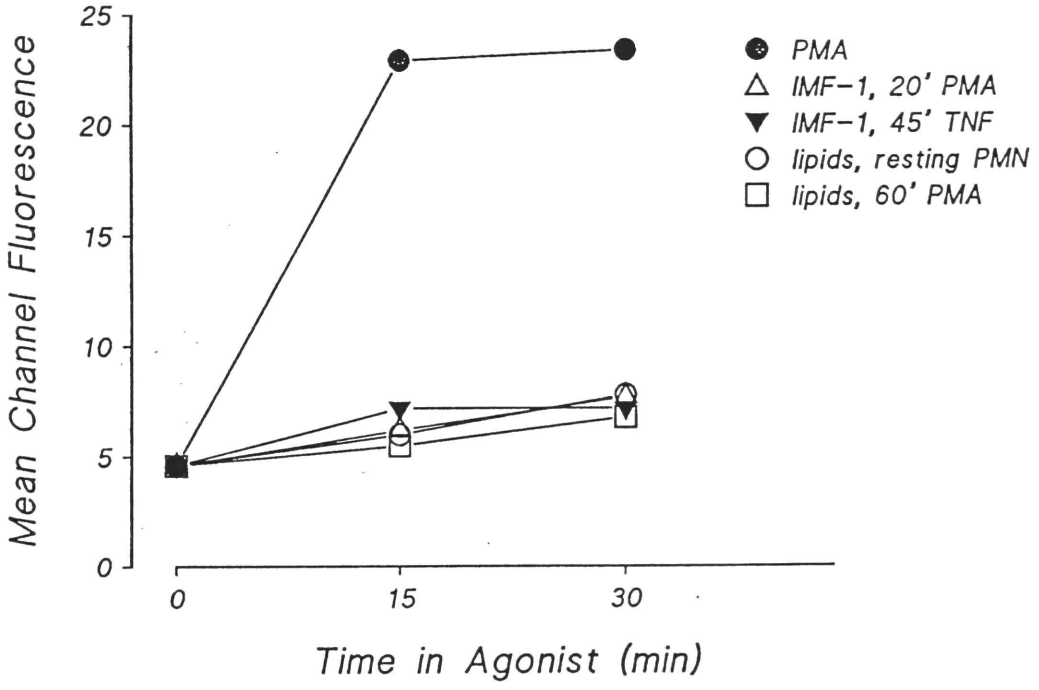



**Figure 27. IMF-1 does not cause degranulation of PMN.**
PMN were incubated with lipid extracts or with PMA (30 ng/ml) for varying times, washed and stained with monoclonal Ab OKM10 (5 μg/ml, 0°C, 20′) to ascertain levels of CR3 expression. Fluorescein-conjugated secondary antibody was then added (10 μg/ml, 0°C, 20′) and cells were analyzed by flow cytometry. IMF-1 derived from cells treated with PMA or TNF (containing 17 U/ml and 42 U/ml IMF-1, respectively) had no effect on CD11b expression. Lipid extracts from cells that lack IMF-1 (lipids from untreated PMN or from PMN treated with PMA 60′) also had no effect on CR3 expression. Identical results were obtained in three separate experiments.

suggest that IMF-1 does not initiate signal transduction as do classical agonists and thus appears to act simply as an allosteric activator of CR3. The more pronounced increase in EC3bi binding (figure 26) triggered by PMA or by IMF-1 after PMA treatment, compared to IMF-1 alone, could be explained by the fact that PMA causes degranulation of PMN, leading to increased cell surface CR3. Since IMF-1 does not cause degranulation it only affects the CR3 already at the surface. Thus IMF-1 has a greater effect on cells already treated with PMA than it does on naive or IMF-1-treated cells.

**IMF-1 affects the ability of CR3 to bind several ligands.**

CR3 binds several ligands in addition to C3bi. PMN bind fibrinogen-coated surfaces via CR3, and this binding can be enhanced by treating PMN with PMA (figure 28) (Wright et al.,1989). Treatment of resting PMN with IMF-1 also enhanced adhesion of PMN to fibrinogen-coated culture surfaces in a transient fashion (figure 28), and this binding was inhibitable with mAbs against CR3 (table XII), suggesting that IMF-1 regulates the ability of CR3 to bind fibrinogen. The experimental design does not rule out the possibility that IMF-1 interacts with fibrinogen rather than CR3 on the PMN. CR3 on PMN also binds lipid IVa, a biosynthetic precursor of lipopolysaccharide (Wright et al.,1988), and this binding can be enhanced by treating PMN with agonists such as NAP1/IL8 (Detmers et al.,1990). Treatment of resting PMN with IMF-1 enhanced binding of erythrocytes which have lipid IVa incorporated into the surface leaflet of the plasma membrane (AI=13 without vs. AI=117 with IMF-1; n=3), suggesting that the ability of CR3 to bind lipid IVa is also regulated

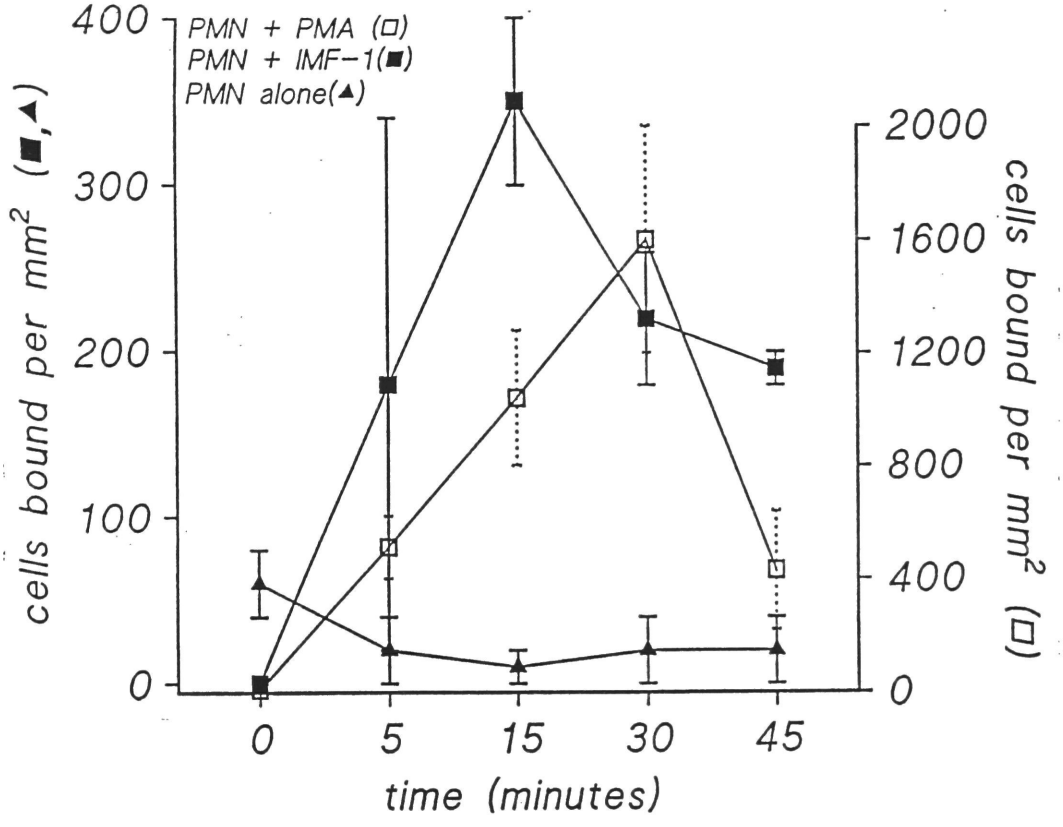



**Figure 28. IMF-1 causes increased binding of CR3 to fibrinogen.**
PMN were allowed to adhere to fibrinogen-coated surfaces at 37°C for varying times in the presence (squares) or absence (triangles) of PMA (30 ng/ml, open squares) or IMF-1 (42 U/ml, partially purified from fNLLP treated cells, solid squares). Data points are the average of two experiments, two wells each.

*IMF-1-INDUCED BINDING OF PMN TO FIBRINOGEN IS CD18-DEPENDENT*

| stimulus | antibody | antigen | cells bound per $mm^2$ |
|---|---|---|---|
| none | | | 200±120 |
| IMF-1 | | | 680±170 |
| IMF-1 | IB4 | CD18 | 220±140 |
| IMF-1 | W6/32 | MHC cl I | 560±220 |

**Table XII.** PMN were incubated in fibrinogen-coated culture wells for 30 minutes at 0°C in the presence of mAbs (10 ng/ml) and then IMF-1 (42 U/ml, isolated from cells treated with fNLLP) was added for 30 minutes at 37°C. Wells were washed with PBS and bound cells were scored by phase contrast microscopy. These data are the average of two experiments, performed in duplicate.

by IMF-1.

CR3, as well as its close homologue, LFA-1, are necessary for the process of diapedesis (Harlan et al.,1985; Anderson and Springer, 1987; Todd and Freyer, 1988; Lo et al.,1989). Treatment of resting PMN with IMF-1 increased their ability to adhere to unstimulated EC (figure 29). IMF-1-induced binding of PMN to EC was dependent on IMF-1 concentration and exhibited transient kinetics (figures 30 and 31). Binding was blocked nearly completely by an antibody against the β chain common to CR3 and LFA-1 (figure 29, IB4) or by a combination of antibodies against the α chains of these two receptors (TS1/22 against LFA-1 α chain, OKM10 against CR3 α chain). Antibodies against either the LFA-1 or CR3 α chain alone blocked only about half of the binding of PMN to EC, as did an antibody against the LFA-1 counter-receptor, ICAM-1. Thus, IMF-1 appears to regulate the ability of both LFA-1 and CR3 to bind structures on the surface of EC.

**IMF-1 modulates the binding activity of LFA-1.**

LFA-1 on lymphocytes also exhibits a regulated binding activity: stimulation with PMA enables LFA-1 on lymphocytes to bind ICAM-1 on adjacent lymphocytes and cause homotypic aggregation (Patarroyo and Makgoba, 1989). Addition of IMF-1 to lymphocytes also caused aggregation of the cells that could be blocked by addition of a mAb directed against the α chain of LFA-1. MAb to CR3 α chain had no effect on the aggregation response (figure 32). Lipids extracted from cells that were not stimulated with agonist and therefore lacked IMF-1 (control lipids, figure 32) could not trigger

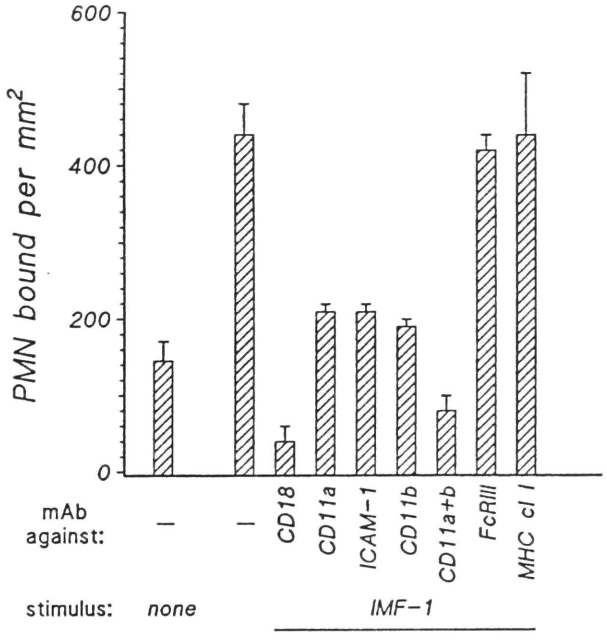



**Figure 29. IMF-1 causes increased binding of PMN to unstimulated endothelium.**

PMN were treated with IMF-1 (42 U/ml, isolated from cells treated with fNLLP), washed, and allowed to bind to unstimulated human umbilical vein endothelial monolayers in the presence or absence of mAbs (10 µg/ml). The mAbs used were OKM10 against CD11b, TS1/22 against CD11a, LB2 against ICAM-1, IB4 against CD18, W6/32 against MHC class I and 3G8 against FcRIII. Nonadherent cells were then washed away and bound cells were quantitated using phase contrast microscopy. Values are the average of duplicate wells, bars show the SEM. This experiment is representative of three separate experiments.

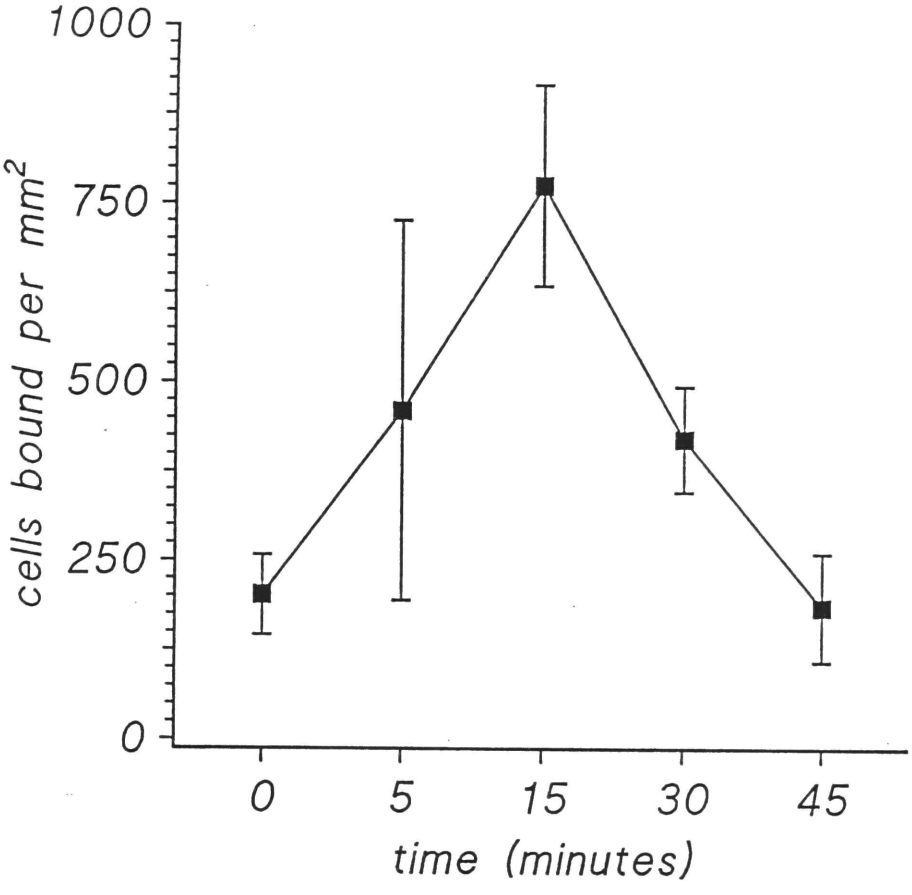



**Figure 30. IMF-1-induced binding of PMN to endothelium is transient.**
PMN were treated with IMF-1 (42 U/ml, isolated from cells treated with fNLLP) for varying times, washed and allowed to bind to unstimulated endothelial monolayers. Values are the average of two experiments, duplicate wells. Bars show the SEM.

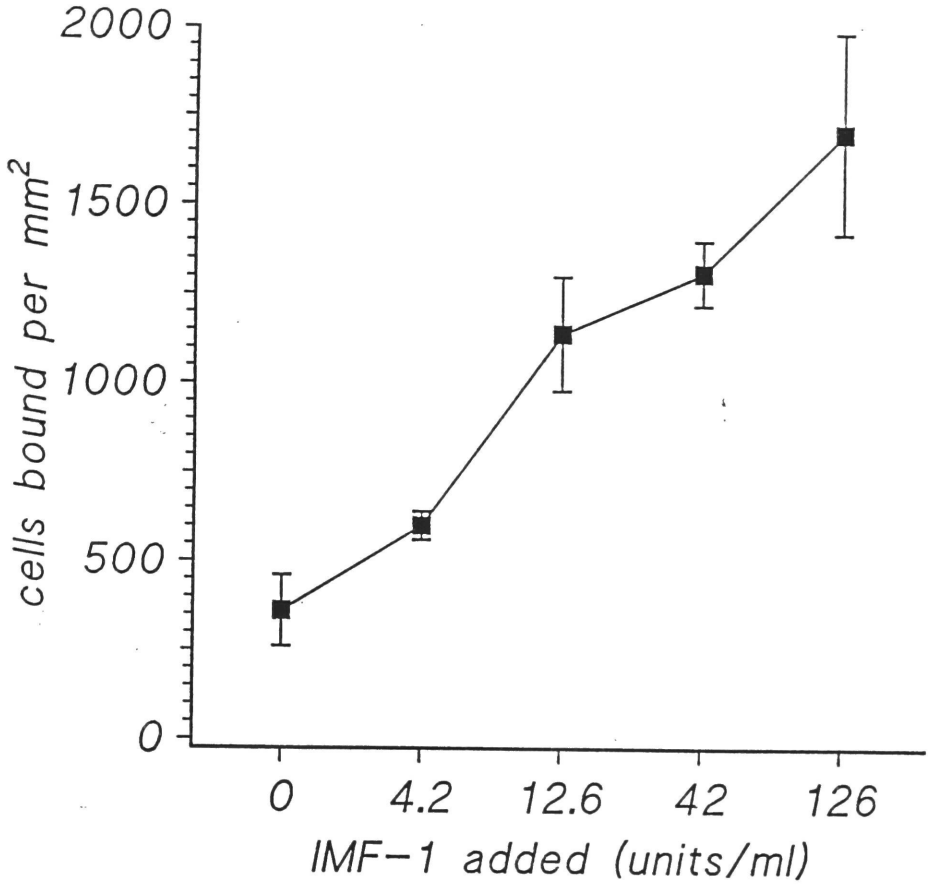



**Figure 31. IMF-1 induces binding of PMN to endothelium in a dose-dependent manner.**
PMN were treated with IMF-1 (isolated from cells treated with fNLLP), washed and allowed to bind to unstimulated endothelial monolayers. Values are the average of two experiments, duplicate wells. Bars show the SEM.

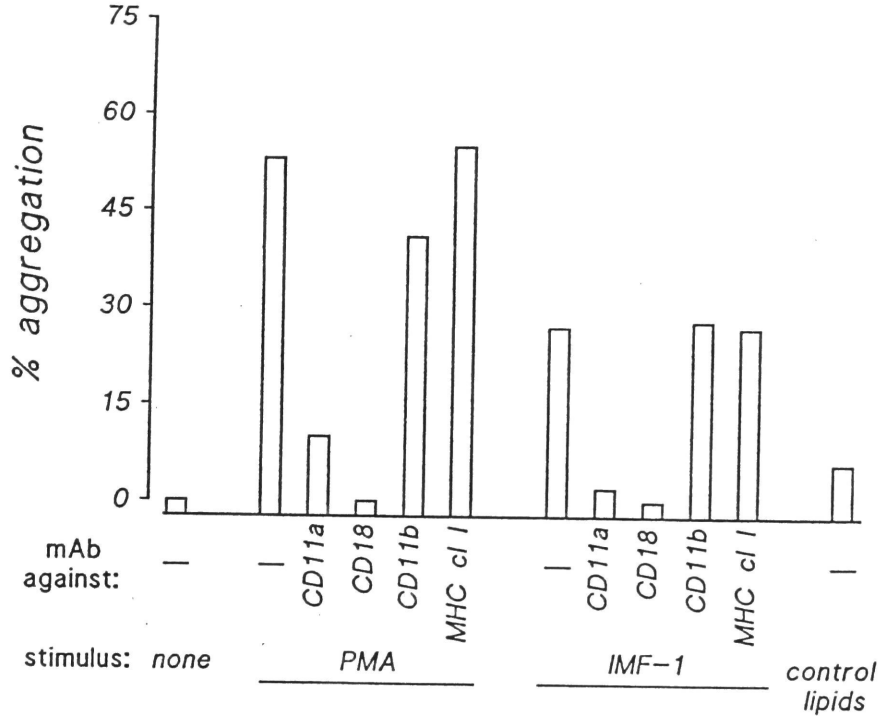



**Figure 32. IMF-1 causes lymphocyte aggregation mediated by LFA-1.** Lymphocytes were purified on Percoll gradients and contaminating monocytes were removed by plastic adherence. Cells were then added to Terasaki plate wells and PMA (100 ng/ml), IMF-1 (42 U/ml) or lipids extracted from resting PMN ("control lipids", extracted from 3 times as many cells as the IMF-1) were added in the presence or absence of antibodies (10 μg/ml). TS1/22 and OKM10 are against CD11a and CD11b, respectively. IB4 is against CD18. W6/32 recognizes MHC class I. Aggregation was determined by phase-contrast microscopy. Similar results were obtained in three separate experiments.

LFA-1-dependent aggregation. Thus, IMF-1 appears to affect the function of both CR3 and LFA-1.

**IMF-1 can be extracted from other leukocytes.**

Lipid extracts derived from monocytes, macrophages and a monocytoid cell line (THP-1) were all found to have IMF-1 activity in the rosetting assay (table XIII). These results suggest that cells of the monocyte lineage, in addition to granulocytes, make IMF-1. It is interesting to note that resting macrophages contain IMF-1 and that these cells also bind EC3bi molecules.

Since lymphocytes are able to respond to the IMF-1 extracted from PMN in a manner similar to their response to PMA, lymphocytes were extracted for lipids to see whether they contain IMF-1, especially after stimulation with PMA. IMF-1 was only recovered in very small quantities from lymphocytes and from an EBV-transformed B cell line (SCHU), and amounts extracted were similar before and after treatment with PMA (table XIII). The lymphocyte lipid extracts were inactive in the lymphocyte aggregation assay and only active at very slight dilution in the rosetting assay for binding to EC3bi, suggesting quantities of IMF-1 present were at or below the threshold of sensitivity for these assays. These results leave open the possibility that although lymphocytes respond to neutrophil-derived IMF-1, they may use a different, IMF-1-like, molecule for regulation of LFA-1, which is not extractable by the IMF-1 purification method. The neutrophil-derived IMF has been designated IMF-*1* to leave open the possibility that other IMFs will be found.

*EXTRACTION OF IMF-1 FROM VARIOUS CELL TYPES*

| | U/$10^7$ cells |
|---|---|
| THP-1, differentiated | 16-25 |
| THP-1, differentiated, + PMA | 5-16 |
| THP-1 | 10 |
| THP-1, membrane fragments and organelles | 3 |
| THP-1, microsomes | 1 |
| macrophages | >1 |
| monocytes | 5 |
| lymphocytes | <0.5 |
| SCHU (B cell line) | <0.25 |
| SCHU + PMA | <0.25 |

**Table XIII.**

Lipids were extracted from at least $10^8$ cells as described in chapter 2. The number of units of IMF-1 in a partially purified lipid extract was determined by rosetting assay.

PMA (30 ng/ml) was added to differentiated THP-1 cells ($5 \times 10^5$/ml) or SCHU cells ($2 \times 10^6$/ml) for 30 minutes at 37°C before harvesting. THP-1 cells ($5 \times 10^5$/ml) were treated with recombinant granulocyte monocyte colony stimulating factor (80 pM) and vitamin $D_3$ ($10^{-8}$ M) for 72 hours at 37°C to induce differentiation. Subcellular fractions of THP-1 cells were kindly provided by Dr. D. Miller (Merck Sharp and Dohme Research Laboratories, N.J.). Subfractionation of homogenized THP-1 cells was performed by spinning cells at 10,000 x g for 10 minutes to remove nuclei and unbroken cells, then spinning at 23,600 x g for 20 minutes to remove organelles and large membrane fragments and 240,000 x g for 60 minutes to remove microsomes.

**Representative β1 and β3 integrins do not respond to IMF-1**

Experiments were performed to test the ability of IMF-1 to affect integrins other than LFA-1 and CR3. Stimulation of platelets with agonists such as thrombin leads to aggregation, due to activation of the ability of the integrin gpIIb/IIIa ($\alpha_{IIb}\beta_3$) to bind soluble fibrinogen. IMF-1 was added to unstimulated platelets to test whether IMF-1 is involved in regulation of IIb/IIIa binding. The cells exhibited no change in aggregation state in response to doses of IMF-1 as high as 100 U/ml (figure 33), although they readily aggregated in response to thrombin. IMF-1 also was unable to convert gpIIb/IIIa to an activated configuration, as determined by an inability of IMF-1-treated platelets to bind PAC1, a mAb that only recognizes activated receptor (table XIV). These data suggest IMF-1 is not responsible for regulation of this $\beta_3$ integrin.

VLAs -4 and -5 on T cells bind more avidly to fibronectin-coated surfaces upon stimulation of the cells with PMA (Shimizu et al.,1990). Exogenous IMF-1 was unable to increase VLA binding to Fn (table XV) suggesting increased binding in response to PMA is not due to IMF-1 production. These results show that IMF-1 itself is not responsible for the regulated binding activity of these representative $\beta_1$ and $\beta_3$ integrins. However, the data do not rule out the existence of other related molecules that could regulate these integrins.

To summarize the biological properties of IMF-1, it is produced monocytes and macrophages as well as by PMN. It increases the binding

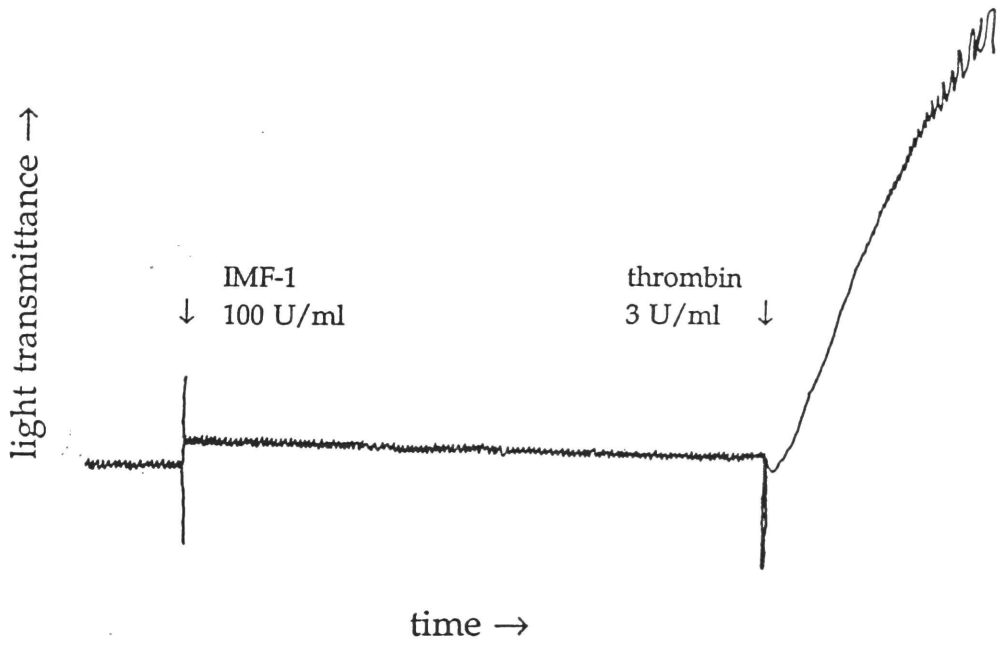



**Figure 33. IMF-1 is not involved in platelet aggregation.**
Aggregation of platelets (2 x $10^8$/ml), as measured by an increase of light transmittance, was monitored in response to 100 U/ml of IMF-1 and 3 U/ml of thrombin.

*IMF-1 does not convert gpIIb/IIIa to an active configuration*

| mAb | stimulus | MFC |
|---|---|---|
| none | none | 13.85 |
| FITC-PAC1 | none | 14.05 |
| FITC-PAC1 | IMF-1 | 14.89 |
| FITC-PAC1 | ctrl lipids | 17.16 |
| FITC-PAC1 | thrombin | 63.70 |

**Table XIV.** Platelets ($10^6$) were incubated for 15', 20°C with IMF-1 (50 U/ml), lipids from unstimulated PMN ("ctrl lipids") or thrombin (0.2 U/ml) and stained with fluorescein-conjugated PAC1 mAb, which only recognizes the $\beta_3$ integrin gpIIb/IIIa in its active form. Platelets were analyzed by flow cytometry. MFC=mean fluorescent channel.

*IMF-1 DOES NOT INCREASE BINDING OF VLA-4 AND VLA-5 TO FIBRONECTIN*

| | cells bound per $mm^2$ |
|---|---|
| lymphocytes alone | 587 ± 46 |
| +10 ng/ml PMA | 2,667 ± 606 |
| +PMA + anti-β1 mAb | 560 ± 80 |
| +PMA + anti-β2 mAb | 2,667 ± 378 |
| +42 U/ml IMF-1 | 667 ± 122 |

**Table XV.** Lymphocytes were allowed to bind to fibronectin-coated surfaces in the presence or absence of PMA or IMF-1 (partially purified from fNLLP-treated PMN). Specificity of binding was verified with mAb AIIB2 against $\beta_1$ and IB4 against $\beta_2$. Adherent lymphocytes were quantified by phase contrast microscopy. These data are the average of three repeats; error is the SEM.

activity of both purified and native CR3 for a variety of ligands, as well as increasing the binding activity of LFA-1. IMF-1 is not secreted by activated PMN, but rather remains cell-associated. IMF-1 appears to have a mode of action distinct from that of neutrophil agonists, and its effect is specific for integrins on PMN, since FcR activity remains unaffected by IMF-1 treatment. While IMF-1 affects leukocyte integrins, it appears to be unable to change the binding activity of $\beta_1$ and $\beta_3$ integrins.

# *Chapter 6 - Discussion*

**Adhesion events are often regulated**

Many adhesion interactions occur only at precise times and locations. Cells must find their place in the developing embryo, mitotic cells must detach from the extracellular matrix during cell division, platelets must aggregate during coagulation and immune cells must extravasate to sites of inflammation or find their infected target and kill it.

Many of the adhesion molecules involved in these interactions have regulated activities. In the course of development, the immunoglobulin superfamily member NCAM is converted from an embryonic form with high sialic acid content, to an adult form with lesser amounts of this sugar, and exhibits a corresponding increase in binding activity (Hoffman and Edelman, 1983). N-Cadherin-mediated adhesion is modulated in chick retinal neurons by an associated N-acetylgalactosaminylphosphotransferase (Balsamo et al.,1991). The LECCAM family member leukocyte adhesion molecule-1 (LAM-1), involved in leukocyte migration, exhibits increased binding activity on lymphocytes and PMN activated with lineage-specific stimuli (Spertini et al.,1991).

Many of the members of the integrin superfamily of adhesion molecules can regulate their ability to bind ligand. Gp IIb/IIIa ($\alpha_v\beta_3$), found on platelets, binds poorly to its substrates until the platelet is activated by a variety of prothrombotic stimuli, such as ADP or thrombin (Plow and Ginsberg, 1989).

VLA antigens ($\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrins) on T cells can bind to extracellular matrix components more efficiently after the lymphocytes are activated (Shimizu et al.,1990). $\alpha_6\beta_1$ on macrophages adheres to laminin after cells are activated with PMA (Shaw et al.,1990). $\alpha_6\beta_1$ in developing retinal neurons can lose its ability to bind laminin during the course of maturation of the chick embryo, although levels of mRNA and protein remain unchanged (de Curtis et al.,1991). Phorbol esters can increase or decrease the binding of $\alpha_5\beta_1$ to fibronectin in CHO or K562 cells, respectively (Danilov and Juliano, 1989; Symington et al.,1989). LFA-1 ($\alpha_L\beta_2$, CD11a/CD18) adhesiveness on T cells is enhanced after TcR crosslinking, treatment with PMA, incubation with mAbs to CD44 or LFA-1 itself, or upon binding of ligand to MHC class II (Dustin and Springer, 1989; Patarroyo and Makgoba, 1989; Koopman et al.,1990; Keizer et al.,1988; Mourad et al.,1990). LFA-1 adhesivity on monocytes is enhanced in response to ligand binding to CD14 (Lauener et al.,1990). The molecular basis of regulated adhesion in integrins has been studied by several laboratories but remains to be elucidated.

**A model for the regulation of integrin function**

Integrins exhibit striking changes in their ability to promote adhesion without corresponding changes in the number of receptors at the cell surface, suggesting that changes in the nature of the existing receptors are responsible for the alterations in adhesivity. Various mechanisms for regulation of receptor function have been postulated. These include aggregation of receptors in the plane of the membrane, phosphorylation or some other covalent

modification of the receptor, changes in the microenvironment of the receptor, or conformational change. Several results suggest that conformational changes underlie the alterations in avidity of integrin receptors. Stimulation of platelets causes a coordinate increase in the avidity of integrin gpIIb/IIIa ($\alpha_{IIb}\beta_3$), and the induction of a neoepitope on the gpIIb/IIIa molecule, suggesting that a conformational change accompanies activation (Coller, 1985; Shattil et al.,1985). The avidity of β1 (Neugebauer and Reichardt, 1991), β2 (Keizer et al.,1988; van Kooyk et al.,1991), and β3 (Kouns et al.,1990; O'Toole et al.,1990) integrins can be strongly enhanced by incubation of cells with certain monoclonal antibodies against the corresponding integrins, suggesting that binding of these monoclonals can induce conformational changes associated with activation. Platelet activation has been shown to lead to a change in the spatial separation or orientation of exoplasmic domains within gpIIb/IIIa, and this change in the receptor may serve to convert this integrin into a functional adhesion receptor (Sims et al., 1991). Epitopes on each of the two chains of gp IIb/IIIa on platelets were labelled with a mAb conjugated to a donor fluorescein or an acceptor tetramethylrhodamine chromophore, and fluorescence resonance energy transfer was measured by flow cytometry. In response to activation, platelets exhibited a 2-fold increase in resonance energy transfer. This increase occured independently of receptor occupancy and was identical regardless of which Ab carried the donor chromophore. Moreover, no energy transfer occured before or after platelet activation when IIb/IIIa was labelled with chromophore-conjugated mAb that recognizes the entire receptor

complex. These data suggest receptors are not clustered: they are not in close enough proximity to transfer energy between subunits on different receptors. Most importantly, the avidity of purified gpIIb/IIIa can be enhanced by pre-treating the purified receptor with peptide ligands (Du et al.,1991), the avidity of purified $\alpha_v\beta_3$ can be altered by reconstituting the purified receptor in liposomes of different lipid compositions (Conforti et al.,1990), and the avidity of purified CR3 can be altered by the addition of the lipid IMF-1. Purified gpIIb/IIIa has been shown to become sensitive to thrombin hydrolysis, and exhibit changes in its intrinsic protein fluorescence, sedimentation coefficient and Stokes radius when treated with synthetic peptides (Parise et al., 1987) that are known to also increase the avidity of the receptor (Du et al., 1991). In these studies, changes in avidity or physical properties are observed with purified receptor, thus making it unlikely that other cellular proteins, phosphorylation events or changes in the receptor microenvironment are necessary for avidity changes and strengthening the notion that changes in receptor conformation are sufficient to alter avidity.

This thesis addresses the mechanism by which the avidity of CR3 is regulated and suggests the following model. In resting cells, CR3 exists in a low avidity state. Upon stimulation with agonists such as PMA, PAF, fNLLP and TNF, PMN synthesize or unmask the lipid IMF-1, which binds to CR3 and effects a conformational change to a high avidity state. Degradation or sequestration of IMF-1 then enables CR3 to relax to a low avidity state. In support of this model, the data demonstrate that the level of IMF-1 present in

cells corresponds to the avidity of their CR3 and that addition of IMF-1 is sufficient to increase avidity of CR3 on the cell surface or in purified form. The data do not rule out the possibility that IMF-1 enhances ligand binding by forming part of the binding site. However, the precedent set by work on other integrins, showing conformational changes upon activation favors the hypothesis that IMF-1 works as an allosteric activator of CR3 and LFA-1. It is likely that the site on the receptor that interacts with IMF-1 is located in the transmembrane domain, due to the hydrophobic nature of IMF-1. Mutational analysis of LFA-1 (Hibbs et al.,1991) has shown that truncation of the β chain cytoplasmic domain results in loss of sensitivity to phorbol esters. The data presented here do not indicate whether association with IMF-1 is the only means by which the conformation of CR3 can be altered or whether conformational changes are the only means by which the avidity of CR3 may be altered. It is possible that other, non-lipid molecules may also cause a conformational change in CR3 to a high avidity state. Moreover, mechanisms other than conformational change could participate in regulation of adhesion. For example, activation of CR3 is tightly associated with clustering of CR3 in the plane of the membrane (Detmers et al.,1987), and clustering of the ligand C3bi on the surface of an erythrocyte is necessary for efficient recognition by CR3 on PMN (Hermanowski-Vosatka et al.,1988). Clustering of receptors may thus function either along with or in lieu of the conformational changes addressed here to affect alterations of adhesivity.

Several workers have observed phosphorylation of CD18, and have

suggested that this phosphorylation event may regulate receptor function (Hara and Fu, 1986; Chatila et al.,1989; Merrill et al.,1990). IMF-1 can increase the avidity of CR3 in the absence of cellular factors, suggesting that direct phosphorylation is not required to change the avidity state of the receptor. In addition, the time course of phosphorylation correlates poorly with that of receptor activity, and only substoichiometric levels of phosphorylation of CR3 (less than 1% of total receptors) can be observed (CB Epstein and SD Wright, unpublished). While these data suggest that phosphorylation does not control ligand binding to receptor, it is possible that phosphorylation of CR3 plays a role in signal transduction by the receptor, or that phosphorylation of a molecule other than CR3 might be involved in triggering the production of IMF-1 or regulating the rate of degradation of IMF-1 in stimulated PMN. In keeping with the possibility that IMF-1 production is enhanced by a phosphorylation event, thio-phosphate loading of PMN, which makes phosphorylation events irreversible, has been shown to cause sustained activation of CR3 (Wright and Meyer, 1986).

Much attention has been focused on the intracellular signals generated by binding of hormones and other molecules to cell surface receptors. IMF-1 may provide an example of a less well studied phenomenon: inside-out signalling. IMF-1 is a molecule which allows the cell to signal to its extracellular milieu in response to intracellular perturbations. Studies of IMF-1 may lend insight into other cellular behaviors of this sort.

IMF-1 is not unique in providing an example of a lipid associating with

and modifying a receptor or channel. Fatty acids have been shown to directly affect the conductance of ion channels in the absence of soluble factors such as nucleotides and calcium (Ordway et al.,1991). The ganglioside $G_{M3}$ can inhibit the EGF-stimulated autophosphorylation of isolated EGF receptor, and phosphatidylethanolamine can enhance this phosphorylation (Bremer et al.,1986). Polysialogangliosides such as $G_{T1b}$ can increase autophosphorylation of purified guinea pig muscle phosphorylase b kinase by 4-10 fold (Chan, 1989). Human and porcine thyrotropin receptors coisolate with a ganglioside similar to $G_{M1}$ (Kielczynski et al.,1991) and the integrins $\alpha_V\beta_3$ and $\alpha_5\beta_1$ coisolate with gangliosides $G_{D2}$ and O-acetylated $G_{D3}$, respectively (Cheresh et al.,1987; Stallcup et al.,1989). However, little evidence exists for cellular the levels of production of these associated lipids.

By regulating the avidity of the leukocyte integrins, IMF-1 can in turn be implicated in the regulation of diapedesis and the evolution of the inflammatory response. Synthesis and degradation pathways for IMF-1 are thus of great interest, and may prove clinically relevant targets for pharmacologic intervention in the control of inflammation.

# *Bibliography*


Albelda, S.M. and C.A. Buck. (1990). Integrins and other cell adhesion molecules. *FASEB J.* **4**:2868.

Anderson, D.C., F.C. Schmalstieg, M.A. Arnaout, S. Kohl, M.F. Tosi, N. Dana, G.J. Buffone, B.J. Hughes, B.R. Brinkley, W.D. Dickey, et al. (1984). Abnormalities of polymorphonuclear leukocyte function associated with a heritable deficiency of high molecular weight surface glycoproteins (GP138): Common relationship to diminished cell adherence. *J.Clin.Invest.* **74**:536.

Anderson, D.C. and T.A. Springer. (1987). Leukocyte adhesion deficiency: An inherited defect in the Mac-1, LFA-1 and p150,95 glycoproteins. *Ann.Rev.Med.* **38**:175.

Auwerx, J. (1991). The human leukemia cell line, THP-1: A multifacetted model for the study of monocyte-macrophage differentiation. *Experien.* **47**:22.

Baggiolini, M., B. Dewald and M. Thelen. (1988). Effects of PAF on neutrophils and mononuclear phagocytes. *Prog.Biochem.Pharmacol.* **22**:90.

Balsamo, J., R. Thiboldeaux, N. Swaminathan and J. Lilien. (1991). Antibodies to the retina n-acetylgalactosaminylphosphotransferase modulate N-cadherin-mediated adhesion and uncouple the N-cadherin-transferase complex from the actin-containing cytoskeleton. *J.Cell Biol.* **113**:429.

Barnstable, C.J., W.F. Bodmer, G. Brown, G. Galfre, C. Milstein, A.F. Williams and A. Ziegler. (1978). Production of monoclonal antibodies to group A erythrocytes, HLA and other human cell surface antigens: new tools for genetic analysis. *Cell* **14**:9.

Bevilacqua, M.P., J.S. Pober, D.L. Mendrick, R.S. Cotran and M.A. Gimbrone. (1987). Identification of an inducible endothelial-leukocyte adhesion molecule. *Proc.Nat.Acad.Sci.USA* **84**:9238.

Bremer, E.G., J. Schlessinger and S.-I. Hakomori. (1986). Ganglioside-mediated modulation of cell growth: Specific effects of $G_{M3}$ on tyrosine phosphorylation of the epidermal growth factor receptor. *J.Biol.Chem.* **261**:2434.

Brown, D.L., D.R. Phillips, C.H. Damsky and I.F. Charo. (1989). Synthesis and expression of the fibroblast fibronectin receptor in human monocytes. *J.Clin.Invest.* **84**:366.

Buyon, J.P., S.B. Abramson, M.R. Philips, S.G. Slade, G.D. Ross, G. Weissman and R.J. Winchester. (1988). Dissociation between increased surface expression of Gp165/95 and homotypic neutrophil aggregation. *J.Immunol.* **140**:3156.

Casals-Stenzel, J., G. Muacevic and K.-H. Weber. (1987). Pharmacological actions of WEB 2086, a new spacific antagonist of platelet activating factor. *J.Pharm.Exp.Ther.* **241**:974.

Chan, K.-F.J. (1989). Ganglioside-modulated protein phosphorylation in muscle: Activation of phosphorylase b kinase by gangliosides. *J.Biol.Chem.* **264**:18632.

Chatila, T.A., R.S. Geha and M.A. Arnaout. (1989). Constitutive and stimulus-induced phosphorylation of CD11/CD18 leukocyte adhesion molecules. *J.Cell Biol.* **109**:3435.

Cheresh, D.A., R. Pytela, M.D. Pierschbacher, F.G. Klier and E. Ruoslahti. (1987). An arg-gly-asp-directed receptor on the surface of human melanoma cells exists in a divalent cation-dependent functional complex with the disialoganglioside GD2. *J.Cell Biol.* **105**:1163.

Chien, S. (1982). Rheology in the microcirculation in normal and low flow states. *Adv.Shock Res.* **8**:71.

Cohnheim, J., Lectures on general pathology: A handbook for practitioners and students, London: The New Sydenham Society, 1889.

Coller, B.S. (1985). A new murine monoclonal antibody reports an activation dependent change in the conformation and/or microenvironment of the platelet glycoprotein IIb/IIIa complex. *J.Clin Invest.* **76**:101.

Conforti, G., A. Zanetti, I. Pasquali-Ronchetti, D. Quaglino Jr., P. Neyroz and E. Dejana. (1990). Modulation of vitronectin receptor binding by membrane lipid composition. *J.Biol.Chem.* **265**:4011.

Cotran, R.S., V. Kumar and S.L. Robbins, Robbins Pathologic Basis of Disease, Philadelphia: W.B. Saunders, Co., 1989. ed. 4

Dana, N., B. Styrt, J.D. Griffin, R.F. Todd III, M.S. Klempner and M.A. Arnaout. (1986). Two functional domains in the phagocyte membrane glycoprotein Mo1 identified with monoclonal antibodies. *J.Immunol.* **137**:3259.

Danilov, Y.N. and R.L. Juliano. (1989). Phorbol ester modulation of integrin-mediated cell adhesion: A postreceptor event. *J.Cell Biol.* **108**:1925.

de Curtis, I., V. Quaranta, R.N. Tamura and L.F. Reichardt. (1991). Laminin receptors in the retina: Sequence analysis of the chick integrin $\alpha_6$ subunit. *J.Cell Biol.* **113**:405.

Detmers, P.A., S.K. Lo, E. Olsen-Egbert, A. Walz, M. Baggiolini and Z.A. Cohn. (1990). Neutrophil-activating protein 1/interleukin 8 stimulates the binding activity of the leukocyte adhesion receptor CD11b/CD18 on human neutrophils. *J.Exp.Med.* **171**:1155.

Detmers, P.A. and S.D. Wright. (1988). Adhesion-promoting receptors on leukocytes. *Curr.Opin.Immunol.* **1**:10.

Detmers, P.A., S.D. Wright, E. Olsen, B. Kimball and Z.A. Cohn. (1987). Aggregation of complement receptors on human neutrophils in the absence of ligand. *J.Cell Biol.* **105**:1137.

Diamond, M.S., D.E. Staunton, A.R. de Fougerolles, S.A. Stacker, J. Garcia-Aguilar, M.L. Hibbs and T.A. Springer. (1990). ICAM-1 (CD54): A counter-receptor for Mac-1 (CD11b/CD18). *J.Cell Biol.* **111**:3129.

Du, X., E.F. Plow, A.L. Frelinger III, T.E. O'Toole, J.C. Loftus and M.H. Ginsberg. (1991). Ligands "activate" integrin $\alpha_{IIb}\beta_3$ (platelet GPIIb-IIIa). *Cell* **65**:409.

Dustin, M.L. and T.A. Springer. (1989). T-cell receptor cross-linking transiently stimulates adhesiveness through LFA-1. *Nature* **341**:619.

Dustin, M.L. and T.A. Springer. (1988). Lymphocyte function associated antigen-1 (LFA-1) interaction with intracellular adhesion molecule-1 (ICAM-1) is one of at least three mechanisms for T lymphocyte adhesion to cultured endothelial cells. *J.Cell Biol.* **107**:321.

English, D. and B.R. Anderson. (1974). Single-step separation of red blood cells, granulocytes and mononuclear phagocytes on discontinuous density gradients of Ficoll-Hypaque. *J.Immunol.Methods* **5**:249.

Fiebig, E., K. Ley and K.-E. Arfors. (1991). Rapid leukocyte accumulation by "spontaneous" rolling and adhesion in the exteriorized rabbit mesentery. *Int.J.Microcirc.Clin.Exp.* **10**:127.

Fleit, H.B., S.D. Wright and J.C. Unkeless. (1982). Human neutrophil Fc γ receptor distribution and structure. *Proc.Nat.Acad.Sci.USA* **79**:3275.

Fogelman, A.M., J. Seager, P.A. Edwards, M. Hokom and G. Popjak. (1977). Cholesterol biosynthesis in human lymphocytes, monocytes and granulocytes. *Biochem.Biophys.Res.Comm.* **76**:167.

Geng, J.-G., M.P. Bevilacqua, K.L. Moore, T.M. McIntyre, S.M. Prescott, J.M. Kim, G.A. Bliss, G.A. Zimmerman and R.P. McEver. (1990). Rapid neutrophil adhesion to activated endothelium mediated by GMP-140. *Nature* **343**:757.

Goldsmith, H.L. and S. Spain. (1984). Margination of leukocytes in blood flow through small tubes. *Microvasc.Res.* **27**:204.

Guindon, Y., Y. Girard, A. Maycock, A.W. Ford-Hutchinson, J.G. Atkinson, P.C Belanger, A. Dallob, D. DeSousa, H. Dougherty, R. Egan, et al. L-651,392: A novel, potent and selective 5-lipoxygenase inhibitor, in Prostaglandin, Thromboxane and Leukotriene Research, Volume 17, edited by B. Samuellson, R. Paoletti and P.W. Ramwell. New York: Raven Press, 1987. p. 554.

Hack, C.E., J. Paardekooper, R.J.T. Smeenk, J. Abbink, A.J.M. Eerenberg and J.H. Nuijens. (1988). Disruption of the internal thioester bond in the third

component of complement (C3) results in the exposure of neodeterminants also present on activation products of C3. *J.Immunol.* **141**:1602.

Hara, T. and S.-M. Fu. Phosphorylation of α,β subunits of 180/100 Kd polypeptides (LFA-1) and related antigens, in Leukocyte Typing II. Volume 3, Human myeloid and Hematopoietic Cells, edited by E.L. Reinherz, B.F. Haynes, L.M. Nadler and I.M. Bernstein. New York: Springer-Verlag, 1986.

Harlan, J.M., P.D. Killen, F.M. Senecal, B.R. Schwartz, E.K. Yee, R.F. Taylor, P.G. Beatty, T.H. Price and H.D. Ochs. (1985). The role of neutrophil membrane glycoprotein GP-150 in neutrophil adherence to endothelium in vitro. *Blood* **66**:167.

Hattori, R., K.K. Hamilton, R.D. Fugate, R.P. McEver and P.J. Sims. (1989). Stimulation of secretion of endothelial cell von Willebrand factor is accompanied by rapid redistribution to the cell surface of the intracellular granule membrane protein GMP-140. *J.Biol.Chem.* **264**:7768.

Henson, P.M., J.E. Henson, C. Fittschen, G. Kimani, D.L. Bratton and D.W.H. Riches. Phagocytic cells: Degranulation and Secretion, in Inflammation: Basic Principles and Clinical Correlates, edited by J.I. Gallin, I.M. Goldstein and R. Snyderman. New York: Raven Press, 1988. pp. 363-390.

Hermanowski-Vosatka, A., P.A. Detmers, O. Gotze, S.C. Silverstein and S.D. Wright. (1988). Clustering of ligand on the surface of a particle enhances adhesion to receptor-bearing cells. *J.Biol.Chem.* **263**:17822.

Hernandez, L.A., M.B. Grisham, B. Twohig, K.-E. Arfors, J.M. Harlan and D.N. Granger. (1987). Role of neutrophils in ischemia-reperfusion-induced microvascular injury. *Am.J.Physiol.* **253**:H699.

Hibbs, M.L., H. Xu, S.A. Stacker and T.A. Springer. (1991). Regulation of adhesion to ICAM-1 by the cytoplasmic domain of LFA-1 integrin β subunit. *Science* **251**:1611.

Hoffman, S. and G.M. Edelman. (1983). Kinetics of homophilic binding by embryonic and adult forms of the neural cell adhesion molecule. *Proc.Nat.Acad.Sci.USA* **80**:5762.

Huang, A., M.B. Furie, S.C. Nicholson, J. Fischbarg, L.S. Liebovitch and S.C. Silverstein. (1988). Effects of human neutrophil chemotaxis across human endothelial cell monolayers on the permeability of these monolayers to ions and macromolecules. J. Cell Phys. **135**:355.

Hynes, R.O. (1987). Integrins: A family of cell surface receptors. *Cell* **48**:549.

Keizer, G.D., W. Visser, M. Vliem and C.G. Figdor. (1988). A monoclonal antibody (NKI-L16) directed against a unique epitope of the α-chain of human leukocyte function-associated antigen 1 induces homotypic cell-cell interactions. *J.Immunol.* **140**:1393.

Kielczynski, W., L.C. Harrison and P.J. Leedman. (1991). Direct evidence that ganglioside is an integral component of the thyrotropin receptor. *Proc.Nat.Acad.Sci.USA* **88**:1991.

Koopman, G., Y. van Kooyk, M. de Graaff, C.J.L.M. Meyer, C.G. Figdor and S.T. Pals. (1990). Triggering of the CD44 antigen on T lymphocytes promotes T cell adhesion through the LFA-1 pathway. *J.Immunol.* **145**:3589.

Kouns, W.C., C.D. Wall, M.M. White, C.F. Fox and L.K. Jennings. (1990). A conformation-dependent epitope of human platelet glycoprotein IIIa. *J.Biol.Chem.* **265**:20594.

Lanier, L.L., M.A. Arnaout, R. Schwarting, N.L. Warner and G.D. Ross. (1985). p150/95, third member of the LFA-1/CR3 polypeptide family, identified by anti-LeuM5 monoclonal antibody. *Eur.J.Immunol.* **15**:713.

Larsen, E., A. Celi, G.E. Gilbert, B.C. Furie, J.K. Erben, R. Bonfanti, D.D. Wagner and B. Furie. (1989). PADGEM protein: A receptor that mediates the interaction of activated platelets with neutrophils and monocytes. *Cell* **59**:305.

Lauener, R.P., R.S. Geha and D. Vercelli. (1990). Engagement of the monocyte surface antigen CD14 induces lymphocyte function-associated antigen-1/intercellular adhesion molecule-1-dependent homotypic adhesion. *J.Immunol.* **145**:1390.

Lawrence, M.B. and T.A. Springer. (1991). Leukocytes roll on a selectin at physiologic flow rates: Distinction from and prerequisite for adhesion through integrins. *Cell* **65**:859.

Lawrence, M.B., C.W. Smith, S.G. Eskin and L.V. McIntire. (1990). Effect of venous shear stress on CD18-mediated neutrophil adhesion to cultured endothelium. *Blood* **75**:227.

Lo, S.K., S. Lee, R.A. Ramos, R. Lobb, M. Rosa, G. Chi-Rosso and S.D. Wright. (1991). Endothelial-leukocyte adhesion molecule 1 stimulates the adhesive activity of leukocyte integrin CR3 (CD11b/CD18, Mac-1, $\alpha_M\beta_2$) on human neutrophils. *J.Exp.Med.* **173**:1493.

Lo, S.K., P.A. Detmers, S.M. Levin and S.D. Wright. (1989). Transient adhesion of neutrophils to endothelium. *J.Exp.Med.* **169**:1779.

Lo, S.K., G. van Seventer, S.M. Levin and S.D. Wright. (1989). Two leukocyte receptors (CD11a/CD18 and CD11b/CD18) mediate transient adhesion to endothelium by binding to different ligands. *J.Immunol.* **143**:3325.

Malech, H.L. and J.I. Gallin. (1987). Neutrophils in human diseases. *New Eng.J.Med.* **317**:687.

Marchesi, V.T. (1961). The site of leukocyte emigration during inflammation. *Q.J.Exp.Physiol.* **46**:115.

Marlin, S.D. and T.A. Springer. (1987). Purified intercellular adhesion molecule-1 (ICAM-1) is a ligand for lymphocyte function-associated antigen-1 (LFA-1). *Cell* **51**:813.

Merrill, J.T., S.G. Slade, G. Weissman, R. Winchester and J.P. Buyon. (1990). Two pathways of CD11b/CD18-mediated neutrophil aggregation with different involvement of protein kinase C-dependent phosphorylation. *J.Immunol.* **145**:2608.

Mourad, W., R.S. Geha and T. Chatila. (1990). Engagement of major histocompatability complex class II molecules induces sustained lymphocyte function-associated molecule 1-dependent cell adhesion. *J.Exp.Med.* **172**:1513.

Neugebauer, K.M. and L.F. Reichardt. (1991). Cell-surface regulation of $\beta_1$ integrin activity on developing retinal neurons. *Nature* **350**:68.

Nobis, U., A.R. Pries, G.R. Cokelet and P. Gaehtgens. (1985). Radial distribuution of white cells during blood flow in small tubes. *Microvasc.Res.* **29**:295.

O'Toole, T.E., J.C. Loftus, X. Du, A.A. Glass, Z.M. Ruggieri, S.J. Shattil, E.F. Plow and M.H. Ginsberg. (1990). Affinity modulation of the $\alpha_{IIb}\beta_3$ integrin (platelet GPIIb-IIIa) is an intrinsic property of the receptor. *Cell Reg.* **1**:883.

Ordway, R.W., J.J. Singer and J.V. Walsh, Jr.. (1991). Direct regulation of ion channels by fatty acids. *Trends in Neurosci.* **14**:96.

Osborn, L. (1990). Leukocyte adhesion to endothelium in inflammation. *Cell* **62**:3.

Parise, L.V., S.L. Helgerson, B. Steiner, L. Nannizzi, and D. R. Phillips. (1987). Synthetic peptides derived from fibrinogen and fibronectin change the conformation of purified platelet glycoprotein IIb-IIIa. *J. Biol. Chem.* **262**:12597.

Patarroyo, M. and M.W. Makgoba. (1989). Leukocyte adhesion to cells: Molecular basis, physiological relevance, and abnormalities. *Scand.J.Immunol.* **30**:129.

Patarroyo, M., E.A. Clark, J. Prieto, C. Kantor and C.G. Gahmberg. (1987). Identification of a novel adhesion molecule in human leukocytes by monoclonal antibody LB-2. *FEBS Lett.* **210**:127.

Philips, M.R., J.P. Buyon, R. Winchester, G. Weissman and S.B. Abramson. (1988). Up-regulation of the iC3b receptor (CR3) is neither necessary nor sufficient to promote neutrophil aggregation. *J.Clin.Invest.* **82**:495.

Plow, E.F. and M.H. Ginsberg. (1989). Cellular adhesion: gpIIb/IIIa as a prototypic adhesion receptor. *Prog.Hemostasis Thromb.* 9:117.

Pober, J.S., M.P. Bevilacqua, D.L. Mendrick, L.A. Lapierre, W. Fiers and M.A. Gimbrone. (1986). Two distinct monokines, interleukin 1 and tumor necrosis factor, each independently induce biosynthesis and transient expression of the same antigen on the surface of cultured human vascular endothelial cells. *J.Immunol.* **136**:1680.

Roberts, M.F., R.A. Deems, T.C. Mincey and E.A. Dennis. (1977). Chemical modification of the histidine residue in phospholipase $A_2$. *J.Biol.Chem.* **252**:2405.

Rouzer, C.A., A.W. Ford-Hutchinson, H.E. Morton and J.W. Gillard. (1990). MK886, a potent and specific leukotriene biosynthesis inhibitor blocks and reverses the membrane association of 5-lipoxygenase in ionophore-challenged leukocytes. *J.Biol.Chem.* **265**:1436.

Sanchez-Madrid, F., A.M. Krensky, C.F. Ware, E. Robbins, J.L. Strominger, S.J. Burakoff and T.A. Springer. (1982). Three distinct antigens associated with human T-lymphocyte-mediated cytolysis: LFA-1, LFA-2 and LFA-3. *Proc.Nat.Acad.Sci.USA* **79**:7489.

Shattil, S.J., J.A. Hoxie, M. Cunningham and L. Brass. (1985). Changes in the platelet membrane glycoprotein IIb/IIIa complex during platelet activation. *J.Biol.Chem.* **260**:11107.

Shaw, L.M., J.M. Messier and A.M. Mercurio. (1990). The activation dependent adhesion of macrophages to laminin involves cytoskeletal anchoring and phosphorylation of the $\alpha_6\beta_1$ integrin. *J.Cell Biol.* **110**:2167.

Shimizu, Y., G. van Seventer, K.J. Horgan and S. Shaw. (1990). Regulated expression and binding of three VLA ($\beta$/1) integrin receptors on T cells. *Nature* **345**:250.

Silverstein, R.L. and R.L. Nachman. (1987). Thrombospondin binds to monocyte-macrophages and mediates platelet-monocyte adhesion. *J.Clin.Invest.* **79**:867.

Simpson, P.J., R.F. Todd III, J.C. Fantone, J.K. Mickelson, J.D. Griffin, B.R. Luccesi, M.D. Adams, P. Hoff, K. Lee and C.E. Roger. (1988). Reduction of

experimental canine myocardial reperfusion injury by a monoclonal antibody (anti-Mo1, anti-CD11b) that inhibits leukocyte adhesion. *J.Clin.Invest.* **81**:624.

Sims, P.J., M.H. Ginsberg, E.F. Plow, and S.J. Shattil. (1991). Effect of platelet activation on the conformation of the plasma membrane glycoprotein IIb-IIIa complex. *J. Biol. Chem.* **266**:7345.

Smith, C.W., R. Rothlein, B.J. Hughes, M.M. Mariscalco, F.C. Schmalstieg and D.C. Anderson. (1988). Recognition of an endothelial determinant for CD18-dependent neutrophil adherence and transendothelial migration. *J.Clin.Invest.* **82**:1746.

Spertini, O., G.S. Kansas, J.M. Munro, J.D. Griffin and T.F. Tedder. (1991). Regulation of leukocyte migration by activation of the leukocyte adhesion molecule-1 (LAM-1) selectin. *Nature* **349**:691.

Springer, T.A. (1990). Adhesion receptors of the immune system. *Nature* **346**:425.

Stallcup, W.B., R. Pytela and E. Ruoslahti. (1989). A neuroectoderm-associated ganglioside participates in fibronectin receptor-mediated adhesion of germinal cells to fibronectin. *Dev.Biol.* **132**:212.

Staunton, D.E., S.D. Marlin, C. Stratowa, M.L. Dustin and T.A. Springer. (1988). Primary structure of intracellular adhesion molecule 1 (ICAM-1) demonstrates interaction between members of the immunoglobulin and integrin supergene families. *Cell* **52**:925.

Symington, B.E., F.W. Symington and L.R. Rohrschneider. (1989). Phorbol ester induces increased expression, altered glycosylation, and reduced adhesion of K562 erythroleukemia cell fibronectin receptors. *J.Biol.Chem.* **264**:13258.

Todd III, R.F. and D.R. Freyer. (1988). The CD11/CD18 leukocyte glycoprotein deficiency. *Hem.Onc.Clin.of N.Amer.* **2**:13.

Tuomanen, E.I., K. Saukkonen, S. Sande, C. Cioffe and S.D. Wright. (1989). Reduction of inflammation,tissue damage, and mortality in bacterial meningitis

in rabbits treated with monoclonal antibodies against adhesion-promoting receptors of leukocytes. *J.Exp.Med.* **170**:959.

van Kooyk, Y., P. Weder, F. Hogervorst, A.J. Verhoeven, G. van Seventer, A.A. te Velde, J. Borst, G.D. Keizer and C.G. Figdor. (1991). Activation of LFA-1 through a calcium-dependent epitope stimulates lymphocyte adhesion. *J.Cell Biol.* **112**:345.

Vedder, N.B. and J.M. Harlan. (1988). Increased surface expression of CD11b/CD18 (Mac-1) is not required for stimulated neutrophil adherence to cultured endothelium. *J.Clin.Invest.* **81**:676.

Vedder, N.B., R.K. Winn, C.L. Rice, E.Y. Chi, K.-E. Arfors and J.M. Harlan. (1988). A monoclonal antibody to the adherence-promoting leukocyte glycoprotein, CD18, reduces organ injury and improves survival from hemorrhagic shock and resuscitation in rabbits. *J.Clin.Invest.* **81**:939.

Webster, R.O., S.R. Hong, R.B. Johnston, Jr. and P.M. Henson. (1980). Biological effects of the human complement fragments C5a and $C5a_{des\ arg}$ on neutrophil function. *Immunopharm.* **2**:201.

Wright, S.D., R.A. Ramos, A. Hermanowski-Vosatka, P. Rockwell and P.A. Detmers. (1991). Activation of the adhesive capacity of CR3 on neutrophils by endotoxin: Dependence on lipopolysaccharide binding protein and CD14. *J.Exp.Med.* **173**:1281.

Wright, S.D., S.K. Lo and P.A. Detmers. Specificity and regulation of CD18-dependent adhesions, in Leukocyte Adhesion Molecules, edited by T.A. Springer, D.C. Anderson, A.S. Rosenthal and R. Rothlein. New York: Springer-Verlag, 1990. pp. 190-207.

Wright, S.D., S.M. Levin, M.T.C. Jong, Z. Chad and L.G. Kabbash. (1989). CR3 (CD11b/CD18) expresses one binding site for Arg-Gly-Asp-containing peptides and a second site for bacterial lipopolysaccharide. *J.Exp.Med.* **169**:175.

Wright, S.D., J.I. Weitz, A.J. Huang, S.M. Levin, S.C. Silverstein and J.D. Loike. (1988). Complement receptor type three (CD11b/CD18) of human

polymorphonuclear leukocytes recognizes fibrinogen. *Proc.Nat.Acad.Sci.USA* **85**:7734.

Wright, S.D. and M.T.C. Jong. (1986). Adhesion-promotin receptors on human macrophages recognize E. coli by binding to lipopolysaccharide. *J.Exp.Med.* **164**:1867.

Wright, S.D. and B.C. Meyer. (1986). Phorbol esters cause sequential activation and deactivation of complement receptors on polymorphonuclear leukocytes. *J.Immunol.* **136**:1759.

Wright, S.D., P.E. Rao, W.C. Van Voorhis, L.S. Craigmyle, K. Jida, M.A. Talle, E.F. Westberg, G. Goldstein and S.C. Silverstein. (1983). Identification of the C3bi receptor on human monocytes and macrophages by using monoclonal antibodies. *Proc.Nat.Acad.Sci.USA* **80**:5699.

Wright, S.D. and S.C. Silverstein. (1982). Tumor-promoting phorbol esters stimulate C3b and C3b' receptor-mediated phagocytosis in cultured human monocytes. *J.Exp.Med.* **156**:1149.

End