The importance of the Mac-1, LFA-1 glycoprotein family in monocyte and granulocyte adherence, chemotaxis, and migration into inflammatory sites: insights from an experiment of nature

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Abstract. The Mac-1, LFA-1 (lymphocyte function-associated 1), p150,95 family of glycoproteins, which share a common β subunit of M_r 95 000, are of widespread importance in leucocyte adhesion reactions. This paper focuses on the role of this glycoprotein family in granulocyte and monocyte adhesion and chemotaxis *in vitro*, and in migration into inflammatory sites *in vivo*. Most findings have been made with granulocytes, but results with monocytes are similar. Some studies have used leucocytes from patients exhibiting a severe or moderate deficiency in expression of this glycoprotein family, which is secondary to a defect in the common β subunit. Patients are susceptible to bacterial infections and have defective pus formation and Rebuck skin-window tests, despite chronic granulocytosis. Granulocytes from such patients exhibit defective adherence to serum albumin and fibronectin-coated glass or plastic, defective orientation and directed migration in response to chemoattractants, and are defective in chemoattractant-stimulated aggregation and hyperadherence. Antibodies to the common β subunit, to the Mac-1 α subunit, and to a lesser extent to the LFA-1 and p150,95 α subunits, inhibit many of the same functional responses by normal cells. In normal granulocytes and monocytes chemoattractants stimulate a five-fold increase in Mac-1 and p150,95 surface expression, by mobilization of a latent, presumably intracellular, pool. Cells from patients are deficient in up-regulation of these molecules but show normal up-regulation of other surface receptors, degranulation and oxidative burst. The hypothesis is presented that Mac-1 and p150,95 regulate or directly mediate the increase in granulocyte and monocyte adhesivity, which is essential for diapedesis, chemotaxis and migration into inflammatory sites.

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Cell surface adherence reactions are of central importance in a wide spectrum of granulocyte, monocyte and lymphocyte functions which contribute to host

defences against infection. Granulocyte and monocyte translocation *in vitro* and mobilization *in vivo* are influenced by the nature of cell–substrate adherence interactions. Studies using time-lapse photography have shown that granulocytes adhere preferentially to vascular endothelium adjacent to a site of inflammation before their diapedesis into tissues (Atherton & Born 1972). This 'directed' adherence is facilitated by by-products of inflammation such as C5a and *N*-formyl-methionyl peptides which bind to specific receptors on granulocytes and monocytes and initiate a sequence of events that enhance cellular adherence (reviewed in Snyderman & Pike 1984 and Tonnesen et al 1984). Much evidence exists that physical properties of endothelial cells or experimental substrates (glass, plastic, albumin, fibrinogen, fibronectin) influence adherence and, secondarily, the extent and direction of cell migration (Wilkinson 1982). Migration towards gradients of chemotactic factors *in vitro* appears to require intermittent adhesion which is sufficiently strong to allow attachment to a substrate but sufficiently localized temporally to allow selective detachment (Wilkinson 1982).

Adhesive interactions are fundamental to other granulocyte and monocyte functions. Specific recognition of opsonized microorganisms is facilitated by membrane receptors for immunoglobulin G (IgG) and for the third component of complement (C3), which mediate adhesion to opsonized microorganisms before the cytoskeletal events leading to endocytosis are triggered. Adhesion mediated by IgG (Fc) receptors can also trigger antibody-dependent killing of target cells, independently of endocytosis. In the absence of opsonins, some microorganisms may adhere to granulocytes and monocytes without undergoing ingestion, or may be phagocytosed inefficiently, depending on the physical properties of the microorganism (Dawson & Mandell 1980).

Many different cell surface proteins are important in these events. Among these, and of ubiquitous importance in the aforementioned granulocyte and monocyte adhesion reactions, and additionally in lymphoid adhesive interactions, are the Mac-1, LFA-1 (lymphocyte function-associated 1) family of glycoproteins (Table 1). These molecules appear to synergize with other receptors or act on their own to regulate or mediate a panoply of leucocyte functional interactions. The wide variety of these functions, and their common dependence on cell adhesion, suggests that the Mac-1, LFA-1 glycoproteins are of general importance in leucocyte adhesion reactions. In this sense, they may be analogous to the adhesion molecules of other tissues, such as the nervous system (N-CAM) or liver (L-CAM).

The thesis of this paper is that these glycoproteins regulate monocyte and granulocyte adherence and chemotaxis *in vitro*, and diapedesis and migration into inflammatory sites *in vivo*. Two types of studies are presented. The first uses monoclonal antibodies (MAb) to these glycoproteins (Sanchez-Madrid et al 1983). MAbs have been obtained which are specific for the αM, αL,

ABLE 1 The Mac-1, LFA-1 family[a]

	Mac-1 (OKM1, Mo1)	*LFA-1*	*p150,95*
ubunits $M_r \times 10^{-3}$)	αM β (170,95)	αL β (180,95)	αX β (150,95)
Cell distribution	Monocytes Macrophages Granulocytes Large granular lymphocytes	Lymphocytes Monocytes Granulocytes Large granular lymphocytes	Monocytes Macrophages Granulocytes
timulation increases urface expression	+	−	+
unctions inhibited by nonoclonal antibodies	Complement receptor type three; Granulocyte adherence, stimulated adherence, spreading, aggregation and chemotaxis	Cytolytic T lymphocyte-mediated killing and T helper cell responses; Natural killing; Antibody-dependent cellular cytotoxicity; Phorbol ester-stimulated lymphocyte aggregation	?

Common features. The β subunits appear identical. The α subunits αM and αL are 35% homologous in sequence. The α and β subunits are non-covalently associated in $\alpha_1\beta_1$ complexes. Both α and β subunits are glycosylated and exposed on the cell surface. All functions shown require divalent cations.

Reviewed in Springer et al (1982), Sanchez-Madrid et al (1983) and Springer & Anderson 1985a,b).

or αX subunits, and thus react only with Mac-1, LFA-1, or p150,95, respectively (Table 1). Another type of MAb reacts with the common β subunit, and hence with all three of these glycoproteins. The other type of study uses cells from patients with a recently discovered heritable deficiency of the entire glycoprotein family. This deficiency is detailed below. Before these studies, we describe a physiologically important property of the Mac-1 and p150,95 glycoproteins: their up-regulation by inflammatory stimuli.

Up-regulation of Mac-1 and p150,95 on granulocytes and monocytes

N-formyl-methionyl peptides such as f-Met-Leu-Phe, which are produced by bacteria, and the C5a anaphylatoxin, a product of complement activation, are chemoattractants for monocytes and granulocytes. These molecules bind with high affinity to specific receptors on these cells (Snyderman & Pike 1984).

In addition to chemotaxis, they trigger increased adherence to surfaces (termed 'hyperadherence'), granulocyte aggregation, the respiratory burst, and the secretion of about 20% of the lactoferrin and cobalamin-binding protein stored in granulocytes (Wilkinson 1982, Gallin 1982). These proteins are stored in the secondary or 'specific' granules of granulocytes. After granulocytes in suspension are stimulated with chemoattractants, electron microscope morphometric analysis demonstrates rapid bipolarization of the cell with the formation of lamellipodia at one end, the loss of about 30% of the secondary granules but no loss of primary granules, and an increase of about 25% in surface area. The increase in area can be accounted for by the fusion of the membrane bilayer surrounding secondary granules with the plasma membrane (Hoffstein et al 1982).

Importantly, the chemoattractant f-Met-Leu-Phe stimulates a marked increase in the amount of Mac-1 and p150,95 expressed on the surface of monocytes (Fig. 1) and granulocytes (Springer et al 1984). Mac-1 and p150,95 increase fivefold on both granulocytes and monocytes (Springer et al 1984 and unpublished). In contrast, the related LFA-1 glycoprotein is not increased. The increases are stimulated by 10^{-8}M f-Met-Leu-Phe, which is within the concentration range for chemotaxis (Wilkinson 1982). The chemoattractant C5a, and the secretagogues phorbol myristyl acetate (PMA) and calcium ionophore A23187 stimulate similar increases in surface expression (Anderson et al 1985). Up-regulation is maximal after 8 min at 37 °C, and is not impeded by inhibitors of protein synthesis (L. Miller et al, unpublished).

Thus, Mac-1 and p150,95 are stored in a latent pool in granulocytes and monocytes, and can be mobilized to the cell surface by chemoattractants (Fig. 2). The fivefold increase shows that the amount of Mac-1 and p150,95 is considerably higher than on the unstimulated cell surface. Furthermore, since the fivefold increase in Mac-1 and p150,95 is accompanied by an increase of only about 25% in granulocyte surface area (Hoffstein et al 1982), the density of Mac-1 and p150,95 in the membrane bilayer of the storage vesicle must be about 16-fold higher than on the cell surface. In granulocytes, the intracellular Mac-1 pool cosediments in sucrose gradients with secondary granules (Todd et al 1984), but further experiments are needed before it can be definitely established whether Mac-1 and p150,95 are stored in the membrane enclosing secondary granules or in some other secretory vesicle. The location of the latent pool in monocytes has not yet been examined.

Mac-1, LFA-1 deficiency disease

Recently, a disease has been recognized in which the Mac-1, LFA-1 and p150,95 glycoproteins are deficient (Springer et al 1984, Anderson et al 1985).

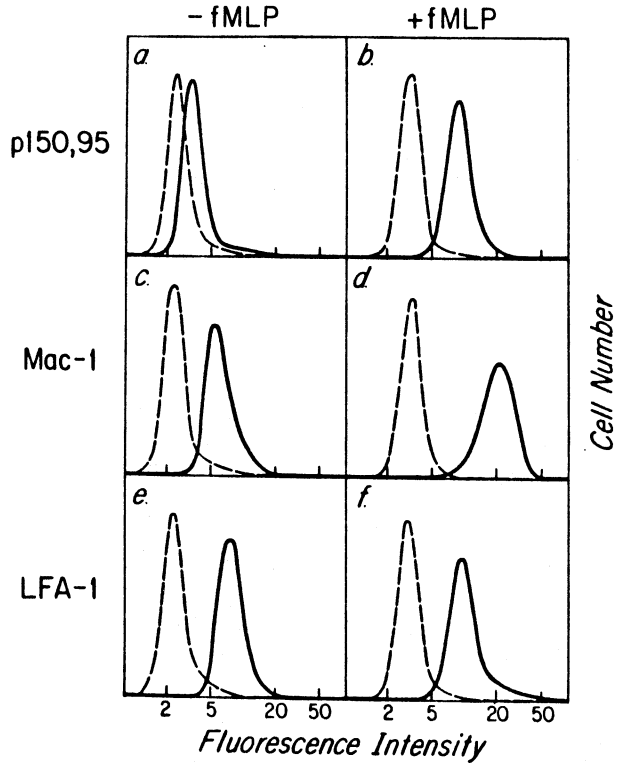

FIG. 1. Chemoattractant stimulates increased expression of p150,95 and Mac-1 but not LFA-1 on monocytes. Mononuclear cells were incubated for 30 min at 37 °C with 10^{-8}M-f-Met-Leu-Phe (fMLP), or held at 4 °C without fMLP as indicated. Cells were stained at 4 °C with specific (solid curves) or control (dashed curves) MAb, followed by fluorescein isothiocyanate anti-mouse IgG, and subjected to immunofluorescence flow cytometry. Both antibodies were used at saturating concentrations, so fluorescence is proportional to the number of antigen molecules per cell. Fluorescence of monocytes was determined by gating on 90° and forward angle light scatter to exclude lymphocytes.

Patients have recurrent, life-threatening bacterial infections, a lack of pus formation, and persistent granulocytosis (Table 2). The deficiency affects all cell lineages which normally express the Mac-1, LFA-1 glycoprotein family, i.e. monocytes, granulocytes and lymphocytes, and cell lines established from patients. Deficiency is inherited as an autosomal recessive mutation. Each of the three α subunits, and the common β subunit, is deficient on the surface of all patients' cells, as shown by immunofluorescent flow cytometry and immunoprecipitation with MAb specific for each subunit (Fig. 3). Two phenotypes

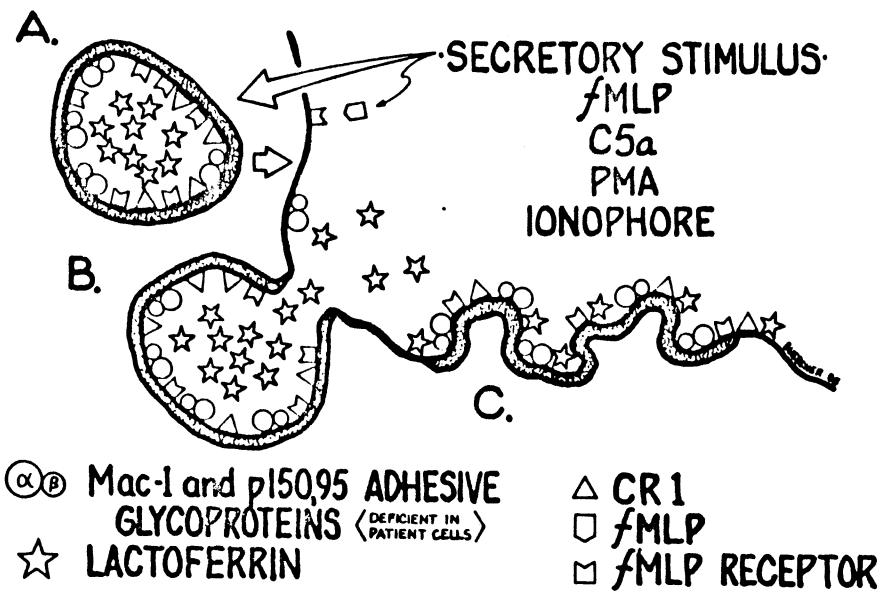

FIG. 2. Secretory vesicle mobilization in granulocytes. The components shown are all mobilized to the cell surface or secreted in response to the indicated stimuli. Deficient cells from patients lack an intracellular pool of Mac-1 and p150,95 and thus fail to mobilize them to the cell surface; the secretory response and mobilization of other surface components such as the CR1 and fMLP receptor is otherwise completely normal in cells from patients. Mac-1 and p150,95 the CR1 and fMLP receptor may be in storage sites distinct from one another and from that of lactoferrin in the secondary granule. They are shown in the same secretory vesicle only for ease of representation. (Drawing by Dr S. Buescher.)

have been defined, severe deficiency and moderate deficiency, with surface expression of <0.2% and 5%, respectively, of the normal amounts of Mac-1, LFA-1, and p150,95 (Fig. 3 and Anderson et al 1985). In both phenotypes, the underlying defect is in the common β subunit (Springer et al 1984), as summarized in Fig. 4. In normal cells, α and β subunit precursors (α' and β') are synthesized which become non-covalently associated, probably in the endoplasmic reticulum, and transported to the Golgi, where carbohydrate processing and a slight increase in relative molecular mass occurs. The mature molecules are then transported to the cell surface or to intracellular storage sites. Cells from patients, however, appear to lack β subunit synthesis or to make it in only small amounts. Normal α' precursors are made but do not undergo carbohydrate processing, suggesting that biosynthesis is blocked before transport to the Golgi apparatus. $\alpha\beta$ association appears to be required

TABLE 2 Clinical features of Mac-1, LFA-1 deficiency syndrome in patients in Texas[a]

	Severe deficiency			*Moderate deficiency*				
Clinical features	*No. 1 F (6 yr)*	*No. 2 F (16 mths)*[b]	*No. 3 F (18 mths)*[b]	*No. 4 M (18 yr)*	*No. 5 M (15 yr)*	*No. 6 M (37 yr)*	*No. 7 M (8 yr)*	*No. 8 F (12 yr)*
Delayed umbilical cord severance and infection	+	+	+	–	–	–	–	–
Persistent granulocytosis (15 000 – 161 000/mm^3)	+++	+++	+++	+	+	+	+	+
Recurrent soft tissue infections:								
Necrotic/ulcerative cutaneous/subcutaneous abscess or cellulitis	+++	+++	+++	++	+	+++	+	+
Perirectal abscess/sepsis	+	+	+	–	–	–	–	–
Mucositis/stomatitis/pharyngitis/ tracheitis	+++	+	++	+	+	+	–	–
Gingivitis/peridontitis	+++	+	+	+++	+++	+++	+++	+++
Pneumonitis	+	+	+	–	–	+	+	–
Peritonitis/necrotizing enterocolitis	+	+	–	–	–	–	–	–
Impaired wound healing	+	+	+	+	+	++	–	–
Parental consanguinity	–	–	+	–	–	±	±	±
Ethnic background	Anglo-Saxon	Hispanic	Iranian	Hispanic	Hispanic	Hispanic	Hispanic	Hispanic

[a] Summarized from Anderson et al (1985).
[b] Deceased.

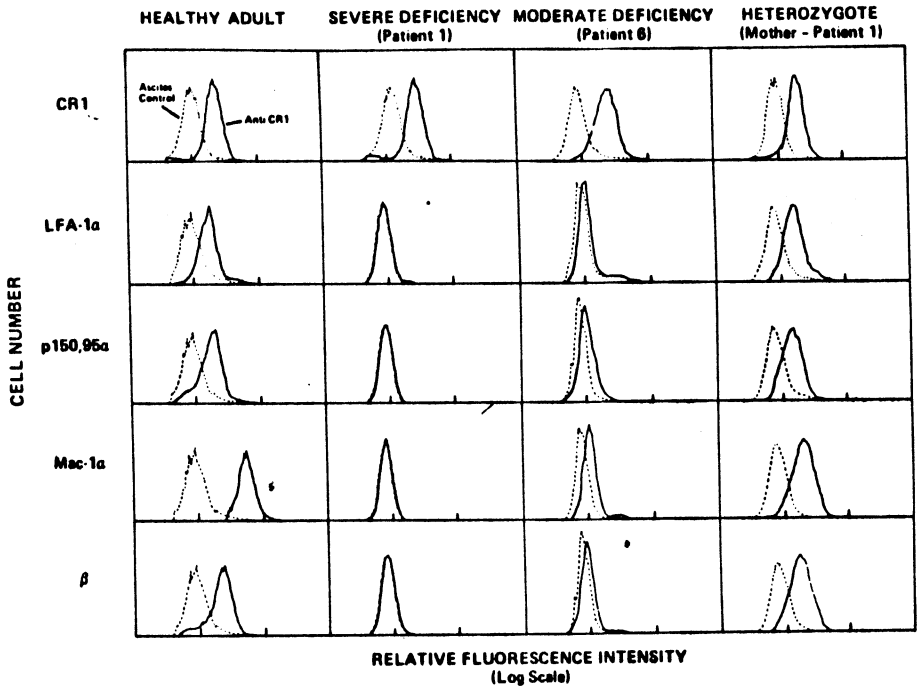

FIG. 3. Immunofluorescence flow cytometry of granulocytes of representative patients with severe and moderate deficiency, a heterozygote, and a healthy adult. Unstimulated granulocytes were indirectly stained with antibodies to the CR1 or the indicated α or β subunits (solid lines) or control MAb (dashed lines). Other methods as in legend to Fig. 1. A similar degree of deficiency was found if granulocytes from patients were stained after f-Met-Leu-Phe stimulation. (From Anderson et al 1985, with permission.)

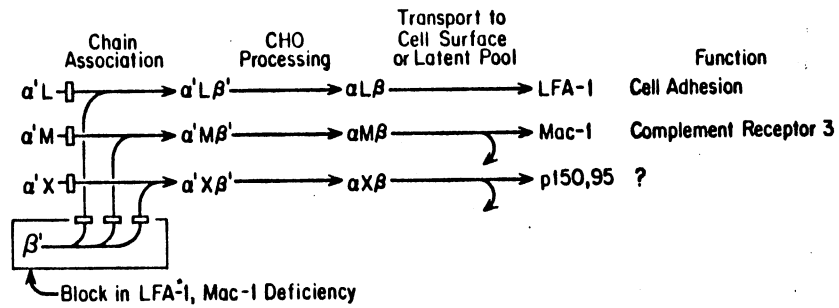

FIG. 4. Biosynthesis of the Mac-1, LFA-1 glycoprotein family (Sanchez-Madrid et al 1983, Springer et al 1984). The evidence for a primary block β subunit synthesis and a secondary block in α subunit processing in cells from patients is described in the text.

for processing and transport to the surface. The α chains are not expressed on the surface (severe deficiency) or are expressed in amounts which appear stoichiometrically limited by the small quantity of β produced (moderate deficiency). In addition to surface expression, granulocytes and monocytes from these patients lack the intracellular pool of Mac-1 and p150,95. After stimulation with f-Met-Leu-Phe or PMA there is little if any increase in Mac-1 and p150,95 surface expression (Springer et al 1984, Anderson et al 1985).

Mac-1, LFA-1 deficiency is a highly specific defect. Granulocytes from patients show normal surface expression of the Fc receptor, the complement receptor type 1 (CR1), and many other markers surveyed in an international monoclonal antibody workshop (Springer & Anderson 1985b). Up-regulation of CR1, secretion of granule constituents such as lactoferrin and lysozyme, the respiratory burst, and superoxide production in response to chemoattractants and PMA are completely normal in cells from patients. Thus, the granule mobilization response depicted in Fig. 2 and other biochemical changes appear normal in such cells, except that Mac-1 and p150,95 are absent from the intracellular compartment that is mobilized to the cell surface. This is in contrast to a distinct disorder involving specific granule deficiency (Gallin et al 1982).

The functional consequences of deficiency

The effects of this deficiency disease have taught us much about the importance of the Mac-1, LFA-1 glycoprotein family in leucocyte adhesion and migration (Anderson et al 1984, 1985). The first known function of Mac-1 was as the complement receptor type 3, which mediates binding and phagocytosis of particles opsonized with the iC3b ligand (Beller et al 1982, Wright et al 1983). Indeed, despite some initial controversy (Dana et al 1983), it is clear that patients are deficient in CR3 (Dana et al 1984, Anderson et al 1984). However, the functional defects are much broader than this.

The recurrent soft tissue infections in patients (Table 2) appear to be due to an inability of granulocytes and monocytes to migrate into inflammatory sites (Anderson et al 1984, 1985). There is no pus formation in the common necrotic, ulcerative skin lesions or in other less readily apparent infected sites. This is confirmed by biopsies, which show that granulocyte mobilization into infected tissues is profoundly impaired. Patients have severe gingivitis, yet a saline wash of the oral cavity reveals no polymorphonuclear leucocytes (PMN), as would be found in other types of gingivitis. Leucocyte mobilization was measured experimentally in patients by the Rebuck skin-window test. The skin was abraded, a coverslip was placed over the site, and granulocytes

and monocytes present in the serous effusions were counted at two-hour intervals. Healthy controls showed immigrating neutrophils at 2 and 4 h followed by monocytes at 6 h. Severely deficient patients showed no mobilization of neutrophils or monocytes to the site even at the 24-hour time point, and leucocyte mobilization in moderately deficient patients was strikingly diminished and delayed. Thus, Mac-1, LFA-1 deficiency results in a profound defect in the ability of leucocytes to leave the circulation by migrating between endothelial cells and through the basement membrane into inflammatory sites, i.e. in diapedesis.

This dysfunction correlates with *in vitro* defects in chemotaxis and adhesion (Anderson et al 1984, 1985). Chemotaxis to f-Met-Leu-Phe and C5a was markedly depressed (Table 3). Patients' granulocytes exhibited a normal bipolar change in shape in suspension in response to chemoattractants, but failed to orient to gradients of f-Met-Leu-Phe or C5a when attached to surfaces (Anderson et al 1984). Granulocytes undergoing orientation to f-Met-Leu-Phe in Zigmond chambers were examined by scanning electron microscopy (Fig. 5). Healthy granulocytes oriented normally, with lamellipodia at their leading edge facing in the direction of the chemoattractant diffusing from the right (Fig. 5B,C). In contrast, patients' granulocytes failed to orient (Fig. 5E,F). Photographs taken in a plane perpendicular to the substrate and parallel to the gradient (Fig. 5C,F) showed that granulocytes from patients were clearly activated, since they were bipolar, but were in a plane perpendicular to the substrate rather than parallel to it (Fig. 5F). They were unable to initiate lateral or peripheral areas of attachment with the substrate.

The orientation and chemotaxis defects appear secondary to a defect in adherence. The percentage of granulocytes adhering to serum-coated glass under 'baseline' conditions was significantly ($P < 0.01$) diminished compared to adherence in healthy controls (Table 3). After stimulation with f-Met-Leu-Phe or PMA, there was little or no increase in adherence by severely deficient PMN and only a modest increase by moderately deficient PMN. In contrast, healthy control PMN demonstrated a normal hyperadherence response. The defect in adherence is also found for glass and plastic coated with other proteins, including fibronectin (Anderson et al 1984, Buchanan et al 1982). Those cells which adhere to protein-coated glass or to plastic demonstrate a profound defect in their ability to spread (Buchanan et al 1982, Anderson et al 1984). C5a, f-Met-Leu-Phe and PMA cause healthy granulocytes to aggregate into clumps, which can be measured in an aggregometer by changes in light transmittance. Deficient granulocytes failed to aggregate (Table 3). Although a change in surface charge on stimulation may contribute to granulocyte aggregation (Gallin 1982), there was no difference in charge between deficient and normal granulocytes (Anderson et al 1984). Phagocytosis of IgG and iC3b-opsonized Oil Red O particles and C3-opsonized zymosan was measured.

Phagocytosis of C3-opsonized particles by deficient granulocytes was diminished (Table 3), as would be expected from the CR3 defect. Deficient granulocytes phagocytosed IgG-opsonized particles normally (Table 3). PMNs demonstrate phagocytosis of many microorganisms in the absence of opsonization, although at a slower rate than after opsonization. Under these conditions, deficient granulocytes demonstrate diminished phagocytosis of some but not all microorganisms. Defects are found for *Staphylococcus aureus* and zymosan (Anderson et al 1984, Thompson et al 1984).

TABLE 3 Assessments of adherence-dependent granulocyte functions[a]

Functional assay	*Severe[b] deficiency*	*Moderate[c] deficiency*	*Healthy adults*
Chemotaxis			
f-Met-Leu-Phe (10^{-8}M)	43 ± 6[d]	66 ± 7	105 ± 4
C5a	42 ± 5	68 ± 14	108 ± 7
Adherence			
Baseline (PBS)	12 ± 2[e]	16 ± 9	38 ± 6
f-Met-Leu-Phe (10^{-8}M)	12 ± 3	28 ± 12	63 ± 6
PMA (5 μg/ml)	16 ± 4	31 ± 12	67 ± 9
Aggregation			
C5a	16 ± 11[f]	15 ± 12	100 ± 0
f-Met-Leu-Phe (10^{-7}M)	16 ± 4	14 ± 13	40 ± 6
PMA (10 μg/ml)	15 ± 9	22 ± 3	105 ± 7
Phagocytosis			
Oil Red O-(IgG)	1.4 ± 0.6[g]	1.4 ± 0.5	1.7 ± 0.4
Oil Red O(iC3b)	1.9 ± 1.2[g]	2.4 ± 1.2	7.0 ± 3.1
C3-opsonized zymosan	4.6 ± 0.7[h]	7.9 ± 3.2	17.4 ± 4.0

[a] Results with each functional assay are represented by mean ± 1 SD value for each patient category, derived from mean values for individual patients in two to six separate experiments. Summarized from Anderson et al (1985).
[b] Includes assessments on severe deficiency patients 1, 2 and 3.
[c] Includes assessments on moderate deficiency patients 4, 6, 7 and 8.
[d] Boyden assay values (mean ± 1 SD) for f-Met-Leu-Phe or C5a (10% zymosan-activated serum) expressed as μm migration/40 min incubation.
[e] Percentage of granulocytes adhering to serum (6%)-coated glass under baseline or stimulated conditions at 21 °C.
[f] Granulocyte aggregation responses to C5a (10% zymosan-activated plasma), f-Met-Leu-Phe or PMA, at 37 °C, measured by the increase in light transmittance and expressed as % of the response to C5a.
[g] Dionylphthalate uptake (μg/10^6 granulocytes in 15 min).
[h] Slope of chemiluminescence evolution (c.p.m.$^2 \times 10^{-5}$).
PBS, phosphate-buffered saline; PMA, phorbol myristate acetate.

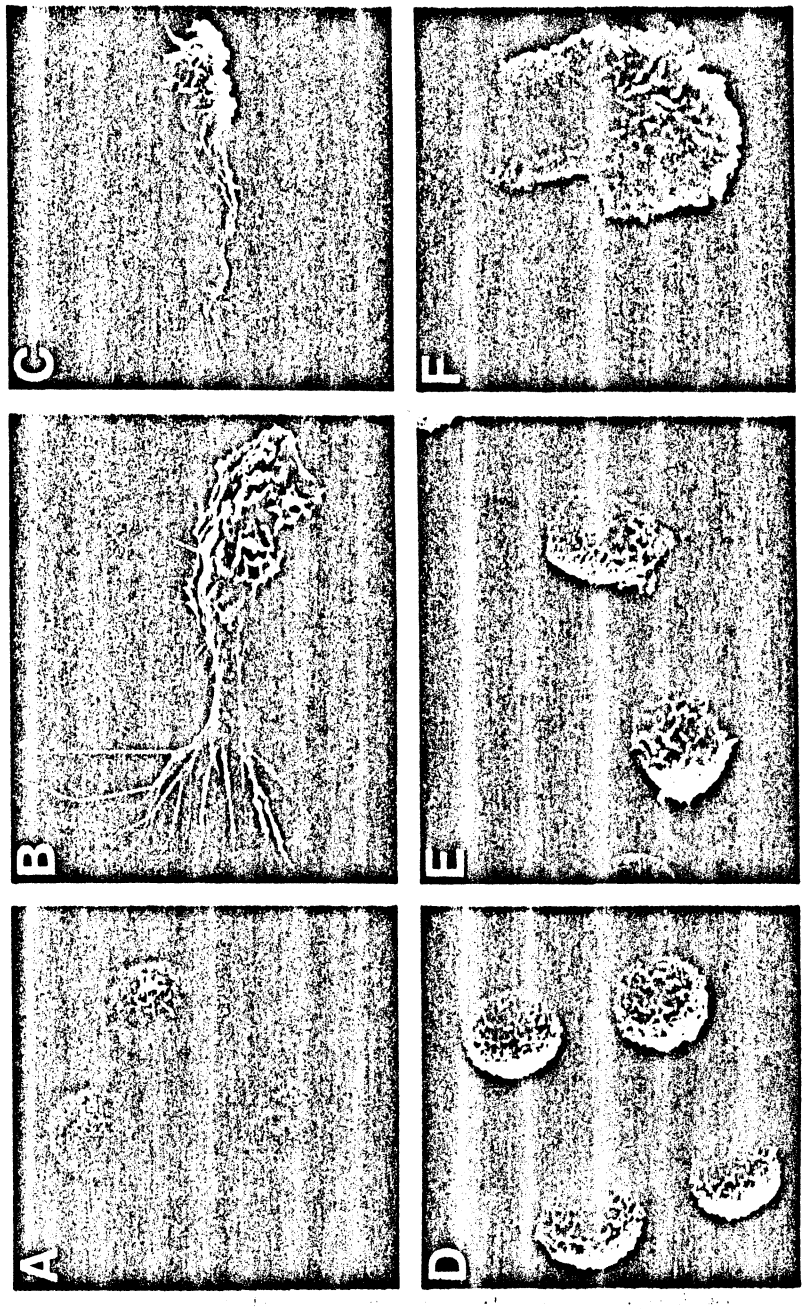

FIG. 5. Orientation of granulocytes from patients or controls in chemotactic gradients. Scanning electron micrographs show the sequence of cell ruffling and spreading on the substrate (A) and achievement of polarity and orientation by healthy adult PMNs (B,C), and parallel experiments on deficient PMNs (D–F). Cells adhere to the coverslip over the bridge in a Zigmond orientation chamber and respond to f-Met-Leu-Phe diffusing from the right. Cells in A, B, D, and E are photographed in a plane parallel to the coverslip, and in C and F perpendicular to the coverslip. (From Anderson et al 1984, with permission.)

Inhibition of adherence-dependent functions by MAb

Binding of monoclonal antibodies to normal granulocytes reproduced the defects found in Mac-1, LFA-1-deficient patients. Both baseline and f-Met-Leu-Phe-stimulated adherence were strikingly inhibited (Fig. 6). Spreading, chemotaxis and aggregation were also inhibited (sumarized in Table 4). The order of potency was anti-β > anti-Mac-1 α > anti-p150,95α > anti-LFA-1. This suggests that all members of the glycoprotein family may contribute to these reactions; the order of potency reflects their relative amounts on the granulocyte surface (Springer & Anderson 1985a). These effects are quite

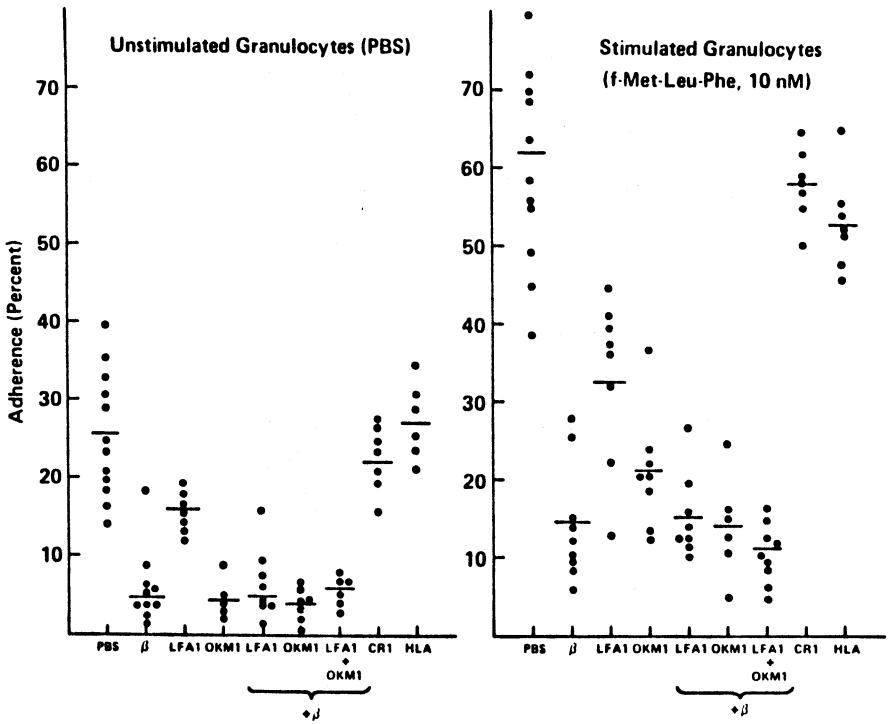

FIG. 6. Effects of monoclonal antibodies to the Mac-1, LFA-1 glycoprotein family on granulocyte adherence. Granulocytes were preincubated with MAbs, washed and then incorporated into Smith Hollers adherence chambers in which they were allowed to adhere to serum (6%)-coated glass substrates under unstimulated (PBS) or stimulated (f-Met-Leu-Phe, 10 nM) conditions at 21 °C. MAb preparations used for the studies shown included: the OKM1 MAb to Mac-1 α (5 µg/ml), F(ab')$_2$ fragments of the TS1/22 MAb to LFA-1 α (5 µg/ml), and F(ab')$_2$ fragments of the TS1/18 MAb to the common β subunit of Mac-1 and LFA-1 (5 µg/ml). Control MAbs included saturating concentrations of an F(ab')$_2$ fragment of rabbit IgG directed against the human C3b receptor (anti-CR1) and an MAb to HLA framework antigen (W6/32).

TABLE 4 Effects of subunit specific monoclonal antibodies to Mac-1 proteins on adherence-dependent and adherence-independent granulocyte functions

	Monoclonal antibody specificity						
Granulocyte function[a]	*OKM1*	*Mac-1 α LM2/1.6*	*LFA-1 α TS1/22 F(ab')$_2$*	*p150,95 α SHCL-3*	*β TS1/18 F(ab')$_2$*	*HLA-A,B W6/32*
Adherence (6% serum-coated glass)	+	+	±	+	+	–
Hyperadherence (6% serum-coated glass, 10 nm fMLP)	+	+	±	+	+	–
Spreading, glass	+*	+*	–	+*	+*	–
Aggregation, C5a	+	+	±	+	+	–
Chemotaxis, C5a	+*		–	±*	+*	–
Phagocytosis (iC3b-ORO)	+		–	–	+	–
Shape change (suspension)	–	–	–	–	–	–
f-Met-Leu-^{3}H-Phe binding	–		–	–	–	–
Superoxide generation (PMA)	–	–	–	–	–	–
Secretion of β-glucuronidase, vitamin B_{12} transport protein (PMA)	–	–	–	–	–	–
Phagocytosis (IgG-ORO)	–	–	–	–	–	–

[a] For most experiments, granulocytes were preincubated in saturating concentrations of MAbs or their F(ab')$_2$ fragments, washed and then assayed.
+ Consistently and significantly blocks function.
± Inconsistent or minimal blockage.
– No blockage.
*Requires presence of monoclonal antibody during assay for inhibitory effect.
ORO, Oil Red O.

specific. They are obtained with F(ab')$_2$ fragments (anti-β and anti-LFA-1) and are not given by IgG MAb bound to other surface molecules (CR1 and HLA-A,B). These MAbs did not inhibit functions that were not deficient in cells from patients, i.e. shape change in suspension, f-Met-Leu-Phe binding, superoxide generation, secondary granule secretion, and phagocytosis of Oil Red O (Table 4).

A dynamic model of chemotaxis and diapedesis

The specific molecular and functional deficits observed in cells from patients suggest that in normal cells adherence and chemotaxis are mediated by the Mac-1, LFA-1 glycoprotein family, and that chemoattractant-stimulated hyperadherence and aggregation are mediated by the increased surface expression of Mac-1 and p150,95. MAb blocking experiments confirm these findings. We believe chemotaxis is profoundly deficient in patients because of the underlying inability of cells to adhere properly to substrates. We propose that, *in vivo*, chemoattractants diffusing from sites of inflammation into the circulation induce Mac-1 and p150,95 up-regulation, leading to increased adherence of monocytes and granulocytes to blood vessels in the inflammatory site (Fig. 7). We further propose that, in analogy to the importance of the Mac-1, LFA-1, and p150,95 glycoproteins in chemotaxis *in vitro*, these glycoproteins mediate essential adherence functions during diapedesis and migration into the inflammatory site. The most important clinical manifestation of Mac-1, LFA-1 deficiency is the inability of granulocytes to migrate into inflammatory sites and form pus. We propose that this is due to a lack of up-regulation of adhesiveness, which is normally regulated by the increased surface expression of Mac-1 and p150,95.

The molecular mechanisms by which these glycoproteins mediate or regulate adhesivity are not known, and are an interesting area for further research. If adhesion is mediated by binding to specific ligands, the experiments with protein-coated glass and plastic substrates suggest that the ligand would first be secreted by the granulocytes or monocytes, then adhere to the substrate, thus allowing attachment of adhesion proteins. It has been demonstrated that the Mac-1 molecule binds at least one specific ligand, iC3b (Wright et al 1983); however, other adhesive interactions mediated by Mac-1 might involve different ligand binding sites or different adhesive mechanisms. It is possible that the Mac-1, LFA-1 glycoproteins each bind several different ligands, or have highly flexible conformations and act as molecular glues, binding to a wide range of molecules. Alternatively, the Mac-1, LFA-1 glycoproteins might not bind ligands directly, but might regulate adhesion through other surface molecules. It should be pointed out that all of the adhesive interactions

in which these molecules participate require metabolic energy and temperatures higher than 4 °C, and thus are not dependent on simple receptor–ligand interactions alone. The Mac-1, LFA-1 glycoproteins may regulate active processes, such as energy-dependent modification of the activity of other molecules, or remodelling of the topography of the plasma membrane.

Whatever the mechanism, the delivery of adhesive proteins to the cell surface in discrete packages, by fusion of secretory vesicles with the plasma membrane, allows an interesting speculation about chemotaxis. Cells undergoing chemotaxis orient in the gradient with lamellipodia at their anterior end and the uropod with retraction fibres at their posterior end (Fig. 5). Sensing of the chemoattractant stimulates not only orientation and motility but also release of secondary granules or some other secretory vesicle containing the Mac-1 and p150,95 glycoproteins. As explained earlier, the concentration of these glycoproteins is much higher in the membrane bilayer of the storage compartment than in the plasma membrane of the unstimulated cell. We propose that during chemotaxis the secretory vesicles containing Mac-1 and p150,95 fuse with the plasma membrane at the leading edge of the cell. The site of fusion is hypothesized to be directed by the same chemoattractant-sensing machinery that guides the ruffling lamellipodia towards the chemoattractant.

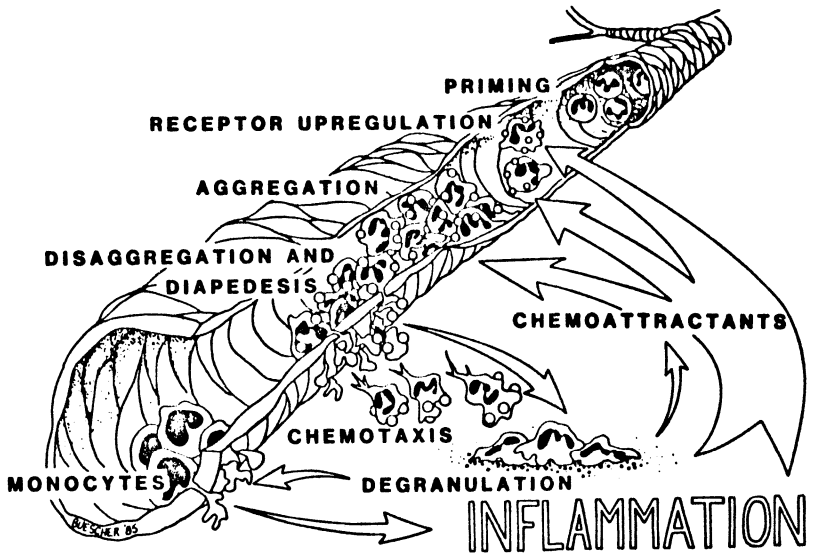

FIG. 7. Chemoattractant-mediated Mac-1 and p150,95 up-regulation, changes in leucocyte adherence, and diapedesis at inflammatory sites. (Drawing by Dr S. Buescher.)

A focal point of high Mac-1 and p150,95 concentration would thus be formed in the plasma membrane at the site of fusion. Adhesion would be initiated or strengthened at this focal point. After further cell translocation, this focal point of attachment would approach the cell uropod. By this time, diffusion of the adhesion proteins in the plane of the membrane, during moments when they are dissociated from ligand, would have lowered their concentration and the strength of adhesion. Alternatively, other time-dependent processes could regulate dissipation of the adhesive force. Detachment in the area of the uropod and its retraction fibres could thus occur, allowing further translocation. Because each granulocyte contains hundreds of specific granules, this cycle could be repeated many times. This process would allow intermittent adhesion sufficiently strong to allow attachment to a substrate but sufficiently localized temporally to allow selective detachment. This process would be superimposed on endocytic recycling of membrane, with endocytosis over the entire surface of the cell, and readdition at the leading edge of the cell. This allows bulk membrane flow from the leading edge to the uropod, and has been hypothesized to effect cell locomotion (Bretscher 1984).

Previous observations are consistent with this model. We found that granulocyte spreading and chemotaxis are inhibited in the continued presence of excess MAb to the Mac-1 α subunit or the common β subunit, but not if cells are pretreated with MAb (Table 4). This is consistent with the idea that these processes require mobilization of Mac-1 from the intracellular latent pool to the cell surface. A role for secondary granule secretion in adherence and chemotaxis has previously been proposed, although it was related to changes in surface charge rather than to Mac-1 and p150,95 up-regulation (Gallin 1982). Chemotaxis through micropore filters is accompanied by secondary granule release, and release is greatest from the granulocytes that migrate furthest. Degranulation occurs at the leading edge of the cell, and has been seen to occur from pseudopodia at points of close apposition to the filter matrix. Furthermore, spreading of neutrophils on glass beads or nylon fibres results in secondary but not primary granule release (Wright & Gallin 1979). Exudate granulocytes accumulating in sterile exudates from heat blisters, or in skin chambers, are selectively depleted in secondary granules, and secondary granule constituents are found in the exudate fluid (Wright & Gallin 1979). Circulating granulocytes from patients with burns are depleted in their stores of secondary granules and deficient in chemotaxis (Gallin 1982). Patients whose granulocyte developmental defect consists of having no secondary granules show a chemotactic defect (Gallin et al 1982). Exposure of granulocytes to step increases in the concentration of f-Met-Leu-Phe induces adhesion sites at the lamellipodia, followed by their redistribution to the uropod, as shown by binding of albumin-coated latex beads (Smith & Hollers 1980). These observations on chemotaxis, adherence and secondary granule release may all be related to chemoattractant-stimulated Mac-1 and p150,95 up-regulation.

More detailed studies of the intracellular location of Mac-1 and p150,95, the coordination of their up-regulation with cell adhesion, orientation and chemotaxis, the formation and dissipation of the adhesive foci, and the structural basis for the adhesion functions of these proteins, promise to provide important insights into the molecular mechanisms of leucocyte chemotaxis and diapedesis.

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DISCUSSION

Singer: Are you suggesting that LFA-1 is in a different granule from Mac-1, as they are not simultaneously expressed?

Springer: Mac-1 and p150,95 are up-regulated and LFA-1 is not. LFA-1 is not stored inside the cell but is expressed only on the cell surface. There are two possible mechanisms for this compartmentalization. One is that the α subunit controls storing, so the LFA-1 α subunit directs its αβ complex only to the cell surface and the other subunits direct their αβ complexes to both the cell surface and the secondary granule. The other possibility concerns the fact that secondary granules form at a very late stage in granulocyte differentiation. The primary granules are formed at the promyelocyte stage and the secondary granules bud off from the Golgi at the myelocyte stage. We know that there is

differential regulation of α subunit expression during granulocyte or myeloid differentiation. The LFA-1 on the cell surface may be synthesized at the promyelocyte stage but at the myelocyte stage no LFA-1 may be made, which would explain why it is not in secondary granules.

Hogg: Götze & Sundsmo (1981) demonstrated that complement components can cause spreading and adhesion of macrophages. Could the complement receptor function of the CR3 molecule therefore be involved in adhesion? Does adhesion perhaps occur via the complement component?

Springer: Götze & Sundsmo (1981) reported that factor B causes spreading of the cells, but factor B does not interact with CR3 so I haven't been able to make any connection between those studies and this work.

Schreiber: Those studies showed that B was ineffective. They reported that activated factor B and C5a were antagonists of one another. Since you are now proposing that chemoattractants increase the expression of this family of proteins it might be worth seeing whether activated factor B decreases the expression of these proteins, as it antagonizes the chemotactic action of complement.

Stanley: What about the role of these antigens in monocyte and macrophage adhesion?

Springer: I think the results with monocytes would be similar to those for granulocytes, because Rebuck skin-window tests show that in both monocytes and granulocytes diapedesis is deficient.

Unkeless: D.T. Fearon (personal communication) looked at Fc receptors on neutrophils and didn't find that the low avidity Fc_γ receptor was mobilized.

Springer: We found the same thing with your 3G8 antibody.

Unkeless: Is there any kind of up-regulation of the Mac-1 and LFA-1 family of monocytes?

Springer: Yes. We see a fivefold up-regulation on blood monocytes of Mac-1 and p150,95.

Nathan: Zanvil Cohn alluded in his opening remarks to the role of monocyte adherence and emigration under the endothelium of large arteries in initiating the fatty streak. Have any of your teenage or older patients shown unexpected absence of fatty streaks in the large vessels at autopsy?

Springer: We haven't had any who died in their teens. One person of 20 died here in England but the family didn't give permission for an autopsy.

Nathan: Do cells in these patients who get granulocyte transfusions mobilize normally? Could a factor other than that expressed in the patients' leucocytes produce this profound deficiency?

Springer: Granulocyte transfusions have only been stop-gap measures in preventing infectious diseases and weren't given to any of the patients I have studied directly. However, on finding that the parents of a patient deceased in 1981 have amounts of Mac-1 α and β on the surface of their granulocytes which

are 68% and 58% of normal, we have typed them as heterozygote carriers of Mac-1, LFA-1 deficiency. Thus in retrospect, their son appears to have had Mac-1, LFA-1 deficiency. The case history is strongly suggestive of this diagnosis. This patient, studied by Drs T. Holbrook, F. Southwick, and T. Stossel, was given leucocyte transfusions. In answer to your question, this patient was converted from negative to positive in the Rebuck skin window by leucocyte transfusion. The failure of diapedesis in his own granulocytes was thus inherent, rather than due to extrinsic factors. Drs A. Fischer and C. Griscelli in Paris have treated the disease successfully by bone marrow transplantation (Fischer et al 1983). The chimeras are healthy.

Singer: Up-regulation may happen very rapidly but chemotaxis has to go on for a very long time. Up-regulation may be of great interest in adhesion and it could be important at the onset of chemotaxis. But if it is involved in adhesion and chemotaxis, these components must be continually appearing at the leading edge of the cell. The burst may have less to do with the ultimate chemotaxis than with its initial onset and with the initial adhesion.

Springer: In up-regulation experiments, we are inducing an artificial situation when we give 10^{-8} M f-Met-Leu-Phe and see a large, immediate response. In contrast, in chemotaxis assays *in vitro* the ligand diffuses very slowly towards the granulocyte. The granulocyte probably meets a very low concentration of ligand and one would expect a small proportion of the secondary granules to be released at a time. I imagine that this cycle recurs continually and there is some evidence for that. If you put two filters in a Boyden chamber and allow chemotaxis to occur, you can separate those two filters and look at the amount of degranulation in the filter where the cells have moved the furthest. The cells that have moved the furthest have released more secondary granular constituents than the other cells (Wright & Gallin 1979).

Singer: Does your monoclonal antibody for LFA-1 inhibit chemotaxis?

Springer: No.

Cohn: Were your studies done in the presence of cytochalasin?

Springer: No.

Gordon: Are there any patients who lack only the Mac-1 and not the whole family? How do you distinguish between the effects of the lack of one molecule versus the lack of all three molecules in relation to these functions? Could you resolve this with antibody-blocking experiments?

Springer: All 20 patients who have been looked at by a number of different groups appear to lack the entire family of glycoproteins. All these molecules are expressed on monocytes and granulocytes, so the only way to sort out the contribution by individual molecules is by antibody blocking experiments on normal cells. The adherence experiments indicate that all the antibodies have inhibited to a certain extent but there is a definite order of inhibition. The anti-Mac-1 and anti-β are strongest, followed by anti-p150, 95. Anti-LFA-1 is

the weakest. This order of inhibition is in rough proportion to the concentration of these molecules on the granulocyte surface. So they may all contribute to adherence. There is quite good evidence that CR3 is inhibited by anti-Mac-1 antibodies and not by anti-LFA-1 antibodies or anti-p150, 95 antibodies.

Werb: Are any of these molecules expressed on either HL60 or U937?

Springer: On uninduced U937 and HL60 we see LFA-1. If we induce with phorbol ester on U937 we see a very dramatic increase in Mac-1 and p150, 95, while LFA-1 remains level. On HL60 there is some induction of Mac-1.

Werb: What is the evidence that Mac-1 or p150, 95 is really a specific granule membrane constituent of the neutrophil?

Springer: Todd et al (1984) showed that when granule fractions are prepared by sucrose density sedimentation, Mac-1 antigen co-sediments in the secondary granule fraction. Gallin and co-workers have obtained similar results and have also shown that patients who have a genetic defect in which they lack secondary granules appear to lack up-regulation of Mac-1 (O'Shea et al 1985).

Werb: HL60 doesn't have secondary granules so I wondered in what form they express Mac-1.

Springer: They do express it on the cell surface.

Cole: Are the patients and the individuals with moderate to low LFA expression heterozygotes?

Springer: No, they are clearly homozygotes. Their parents have 50% of normal surface expression. The moderately deficient patients have only 5% surface expression.

Cole: Are the β subunits identical by criteria other than immunological cross-reactivity?

Springer: Yes, in the mouse we demonstrated identity by peptide mapping. In humans they are also identical by isoelectric focusing.

Cole: Are you certain that there is one α subunit and one β subunit per unit of LFA or Mac-1?

Springer: Yes. We use a bifunctional cross-linking reagent and we wind up with a cross-linked heterodimer of M_r about 260000. When we break the cross-links we get the α and β subunits. If we had an $\alpha_2\beta_2$ complex we would get something of twice that M_r. We see absolutely no species at the 520000 position or at any other position greater than 260000 M_r.

Cole: Can defects of these kinds of proteins be acquired or are they always due to a genetic defect?

Springer: There is no evidence that they are acquired.

Nathan: There are reports that ethanol and aspirin ingestion decrease neutrophil adherence (MacGregor et al 1974). Would these have any effect on the function of LFA *in vitro*?

Springer: We haven't tested that.

Cohn: Does that happen by acetylation?

Nathan: I don't know. There are clear-cut effects *in vitro* after *in vivo* or *in vitro* treatment.

Springer: Ethanol inhibits the up-regulation. We learnt that as a result of doing the proper control for adding cytochalasin dissolved in ethanol.

Sorg: Did you test adherence in endothelial cell cultures? In other words, what is the natural substrate of adherence of protein coating?

Springer: The Seattle group have similar patients and looked directly at adherence to endothelial cells, which was deficient (Beatty et al 1984).

Cohn: Are there substrates to which these cells attach normally?

Springer: Yes, they adhere well to uncoated glass or plastic. That is a kind of irreversible adhesion which is not dependent on divalent cations and which does not allow chemotaxis. Cells also spread normally on IgG-coated substrates.

Cohn: Presumably via the Fc receptor. Is there specificity in terms of types of protein that you use to coat the glass?

Springer: No, you can use serum albumin, which is not glycosylated, ruling out lectin-like activity. You can also use whole serum, fibrinogen and fibronectin and get equivalent results.

Cohn: Have you used defatted albumin or just fatty acid-containing albumin?

Springer: We used fatty acid-containing albumin.

Singer: You say that calcium is required for adhesion. Is it required for neutrophil motility in a chemotactic gradient?

Springer: We haven't looked at that but it is well documented by Gallin & Rosenthal (1974) and others that magnesium is required for both adhesion and chemotaxis.

Nicola: In the same time course the same chemotactic peptide, f-Met-Leu-Phe, down-regulates granulocyte colony-stimulating factor receptors on human blood neutrophils. That occurs within 15–20 minutes over the range 10^{-9}–10^{-7} M. The amount of down-regulation is about 50% and then the response reaches a plateau (N.A. Nicola et al, unpublished). At the same time as this chemotactic peptide is up-regulating some receptors, it is also down-regulating at least one other, and other more complicated interactions may be going on.

Springer: Fearon and co-workers (Changelian et al 1985) have shown for complement receptor type 1 that after an initial up-regulation, phorbol esters can stimulate down-regulation. That may be due to increased endocytosis.

Whaley: Do patients with a moderate deficiency of CR3 show the same biosynthetic defects as those with severe deficiency?

Springer: Yes, we have seen the same β subunit defect in moderately deficient and severely deficient patients. Both have normal α subunits, which were also normal by isoelectric focusing. We don't have any direct evidence for this

but our model for the difference between moderately and severely deficient patients is that the moderates synthesize about 5% of the normal amount of the β subunit, which stoichiometrically limits the amount that appears on the cell surface.

Cohn: Are both molecules on the cell surface?

Springer: In moderately deficient patients both subunits are expressed on the cell surface. If you immunoprecipitate with anti-LFA antibody, for example, you pull down the β chain as well, so moderately deficient patients have αβ complexes.

Cohn: What do you think the non-covalent linkage is?

Springer: Typical hydrophobic bonds, etc.

Singer: Do the macrophages of these patients show normal chemotaxis? It would be interesting to know to what extent macrophage motility and neutrophil motility are related in their mechanisms.

Springer: We haven't looked at macrophages. Don Anderson may have looked at monocytes.

Nathan: The absence of monocytes in the Rebuck window doesn't necessarily establish an intrinsic monocyte defect. Monocytes may depend on the prior accumulation of neutrophils to respond. One of John Gallin's patients with absent neutrophil specific granules had defective neutrophil and monocyte accumulation in Rebuck windows but the monocytes showed normal chemotaxis *in vitro* while the neutrophils did not (Gallin et al 1982).

Springer: I have seen reports that in the absence of neutrophils there is still an influx of monocytes, i.e. monocyte emigration is normal in patients with neutropenia (Dale & Wolff 1971).

Nathan: This may be due to a quantitative versus a qualitative difference.

Roos: We have a patient with a few per cent of the normal amount of LFA-1 in the lymphocytes. We find normal K and NK function, but this can be inhibited with very small quantities of antibody against the LFA-1 antigen. The amount of LFA-1 on the patient's T cells is apparently enough to give normal function, but it is on the borderline of what is necessary.

Springer: We have similar observations. We have been using target cells which are LFA-1 positive and in moderately deficient patients part of the inhibitory effect is due to binding of LFA-1 antibody to target cell LFA-1. For one of the severely deficient patients we see inhibition which is due to binding to target cell LFA-1. So it appears that LFA-1 can mediate bidirectional interactions between effector and target cells (Krensky et al 1985).

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Interaction and regulation of macrophage receptors

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Abstract. Macrophages express distinct plasma membrane receptors for different isotypes of immunoglobulin, bear at least two receptors for cleaved third complement component (CR1 and CR3) and have a lectin-like receptor that mediates endocytosis of glycoproteins or glycoconjugates with terminal mannose or fucose residues (MFR). Interferon-γ, a macrophage-activating factor, induces effects common to other interferons as well as having unique effects on cell function. The down-regulation of MFR, induction of IgG2a Fc receptors and Class II antigens and enhanced production of superoxide and hydrogen peroxide can be considered interferon-γ-specific effects on macrophages.

Previous reports described synergism of various interferon preparations in anticellular and antiviral effects. However, interferon-α/β can selectively antagonize the down-regulation of macrophage MFR by interferon-γ. The macrophage MFR and CR3 also play a synergistic role in the uptake of zymosan and *Leishmania donovani* in the absence of serum. The receptors may act independently or in concert. Cleaved third complement components can be specifically eluted from zymosan particles in the absence of exogenous complement and are derived from the macrophages themselves. These studies indicate a role for macrophage complement in local opsonization of pathogens at extravascular sites and focus on the role of the tissue macrophage in first-line host defence.

1986 Biochemistry of macrophages. Pitman, London (Ciba Foundation Symposium 118) p 127-136

All cells involved in antigen recognition are required to recognize a wide variety of different antigens. For lymphocytes, this challenge is met by clonal proliferation of T and B cells which possess disulphide-linked heterodimers which confer antigen specificity. By contrast, the repertoire of antigens recognized by mononuclear phagocytes is dependent on exogenous opsonins such as antibody and complement or on the presence of ubiquitous molecules on antigens, for example carbohydrates, specifically mannose. Macrophages are unique in that they express distinct Fc receptors for different isotypes of immunoglobulin (Unkeless et al 1981), bear at least two distinct receptors for fragments of activated third complement component (C3) (Ross et al 1982), and