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Leukocyte Complement Receptors and Adhesion Proteins in the Inflammatory Response: Insights from an Experiment of Nature

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Synopsis

The complement receptor type 3 (CR3) mediates phagocytosis and degradation of iC3b-opsonized particles by macrophages and granulocytes. The CR3 is identical to the Mac-1 molecule, which is composed of two non-covalently associated glycoprotein subunits, αM of M_r 170000 and β of M_r 95000. Patients with recurring, life-threatening bacterial infections have been identified who have moderate (95%) or severe (> 99%) deficiency of Mac-1 and of the related LFA-1 and p150,95 molecules. The primary defect is in the shared β subunit of these molecules. Patient leukocytes are not only deficient in CR3 but in a wide variety of adhesion-dependent functions, including granulocyte chemotaxis, adherence to surfaces, and aggregation. Monoclonal antibodies to the Mac-1 α subunit and to the β subunit block these functions. The hypothesis will be advanced that Mac-1 functions dually as the CR3 and in 'nonspecific' adherence reactions. Adherence functions are stimulated in normal granulocytes by chemoattractants, which also induce a rapid 5-fold increase in Mac-1 and p150,95 on the cell surface. It is proposed that absence of Mac-1 and p150,95 expression and upregulation by patient granulocytes is causally related to their inability to extravasate and migrate into inflammatory sites.

Introduction

Cell surface adherence reactions are of central importance in a wide spectrum of granulocyte, monocyte, and lymphocyte functions which contribute to host defense against infection. Granulocyte and monocyte translocation *in vitro* and mobilization *in vivo* are influenced by the nature of cell–substrate adherence interactions [1]. Studies employing time-lapse photography have shown that granulocytes adhere preferentially to vascular endothelium adjacent to a site of inflammation prior to their diapedesis into tissues [2]. This 'directed' adherence is facilitated by byproducts of inflammation such as C5a which bind to specific receptors on granulocytes and monocytes and initiate a sequence of events that enhance cellular adherence [3].

Adhesive interactions are fundamental to other granulocyte and monocyte

Table 1. *The Mac-1, LFA-1 family*

Common features: the β subunits appear identical. The α subunits αM and αL are 35% homologous in sequence. The α and β subunits are noncovalently associated in $\alpha_1\beta_2$ complexes. Both α and β subunits are glycosylated and exposed on the cell surface. All functions shown require divalent cations. An important property of selected proteins of the Mac-1 family, as well as of other critical leukocyte receptors (e.g. CR1) is the capacity of inflammatory stimuli to 'upregulate' or enhance their surface expression (reviewed in [9, 10]).

Property	Mac-1 (OKM1, Mo1)	LFA-1	p150.95
Subunits	αM, β	αL, β	αX, β
($M_r \times 10^{-3}$)	(170, 95)	(180, 95)	(150, 95)
Cell distribution	Monocytes, macrophages, granulocytes, large granular lymphocytes	Lymphocytes, monocytes, granulocytes, large granular lymphocytes	Monocytes, macrophages, granulocytes
Stimulation increases surface expression	+	−	+
Functions inhibited by MAbs	CR3; 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	?

functions. Specific recognition of opsonized mircro-organisms is facilitated by membrane receptors for IgG and for the third component of complement (C3), which mediate cell–microbe adhesion prior to triggering of endocytosis. Adhesion mediated by IgG (Fc) receptors is also required for antibody-dependent cytotoxicity of target cells. In the absence of opsonins, some micro-organisms may adhere to granulocytes and monocyte–macrophages without undergoing ingestion or may be phagocytosed non-specifically, depending on the physical properties of the micro-organism.

Many different cell surface proteins are important in these events. Increasing evidence indicates that the Mac-1, LFA-1 family of glycoproteins (Table 1) are of ubiquitous importance in the aforementioned granulocyte and monocyte adhesion reactions, and additionally in lymphoid adhesive interactions such as those required for T lymphocyte or natural killer cell cytotoxicity. These molecules appear to synergize with other receptors or function independently to regulate or mediate a panoply of 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 leukocyte adhesion phenomena, and thus, may be analogous to the adhesion molecules of other tissues, such as the nervous system (N-CAM) or liver (L-CAM).

Importantly, chemotactically relevant concentrations of the chemoattractants fMet-Leu-Phe and C5a, and the secretagogues phorbol myristate acetate and calcium ionophore, stimulate a 5–10-fold increase in the amount of Mac-1 and

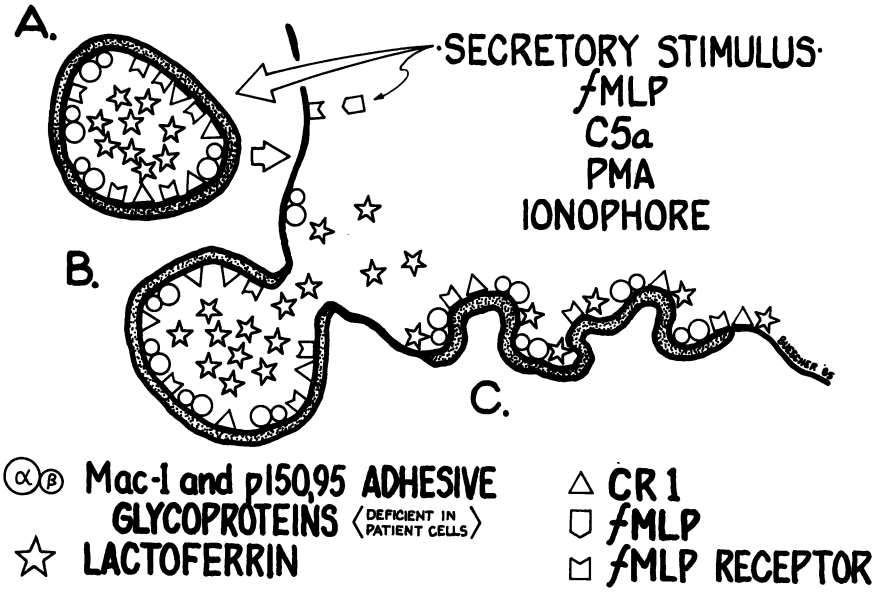

Fig. 1. *Secretory vesicle mobilization in granulocytes*

The components shown are all mobilized to the cell surface or secreted in response to the indicated stimuli. Deficient patient cells 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 fMet-Leu-Phe (fMLP) receptor is otherwise completely normal in patient cells. Mac-1 and p150,95, the CR1, and fMet-Leu-Phe 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.)

p150,95 expressed on the surface of monocytes and granulocytes [4]. In contrast, the related LFA-1 glycoprotein is not increased. 'Upregulation' is maximal after approx. 8 min at 37 °C, and is not impeded by inhibitors of protein synthesis (T. Springer, L. Miller & D. Anderson, unpublished work). Thus, Mac-1 and p150,95 are stored in a latent pool in granulocytes and monocytes, and can be rapidly mobilized to the cell surface by inflammatory stimuli (Fig. 1). In granulocytes, the intracellular Mac-1 pool co-sediments in sucrose gradients with secondary granules [5,6, T. Springer & N. Borregaard, unpublished work]. Furthermore, permeabilization of granulocytes with saponin greatly increases the amount of fluorescent staining with anti-Mac-1 MAb (T. Springer, unpublished work). Further experiments are needed before it can be definitively established whether Mac-1 and p150,95 are stored in the membrane-enclosing secondary granules or some other secretory vesicle. By a number of different measurement techniques, the amount of Mac-1 stored in the latent pool is 5–10-fold higher than on the resting cell surface. Since a 5–10-fold increase in surface Mac-1 is accompanied by only about 25% increase in granulocyte surface area [7], the density of Mac-1 and p150,95 in the membrane bilayer of the storage vesicle must be approx. 16–36-fold higher than on the cell surface.

A major objective of the present paper is to provide evidence that the Mac-1 glycoproteins regulate monocyte and granulocyte adherence, chemotaxis and

other adherence-dependent functions *in vitro*, and are required for leukocyte recruitment into inflammatory sites *in vivo*. Two types of studies are presented. The first utilizes leukocytes from patients with a recently discovered heritable deficiency of the entire Mac-1 glycoprotein family [8]. The other type utilizes MAbs to these glycoproteins [9] together with normal leukocytes *in vitro*. A number of MAbs have been obtained which are specific for the αM, αL, or αX subunits, and thus react only with Mac-1, LFA-1, or p150,95, respectively. Another type of MAb reacts with the common β subunit, and hence with all three of these glycoproteins (Table 1).

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 [4,8]. Patients have recurrent, life-threatening bacterial infections, a lack of pus formation, and persistent granulocytosis. 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 from the surface of all patients' cells, as shown by immunofluorescent flow cytometry and immunoprecipitation with MAb specific for each subunit. Two phenotypes have been defined, severe deficiency and moderate deficiency, with surface expression of $< 0.2\%$ and 5–10%, respectively, of normal amounts of Mac-1, LFA-1, and p150,95 [8] (Fig. 2). Patient granulocytes show normal surface expression of the Fc receptor, the complement receptor type 1 (CR1), and many other markers surveyed in an inernational monoclonal antibody workshop [10].

In both phenotypes, the underlying defect is in the common β subunit [4], as summarized in Fig. 3. In normal cells, α and β subunit precursors (α' and β') are synthesized and become noncovalently associated, probably in the endoplasmic reticulum, and transported to the Golgi, where carbohydrate processing and a slight increase in molecular weight occurs. The mature molecules are then transported to the cell surface or to intracellular storage sites. Patient cells, however, appear to lack β subunit synthesis, or to make it only in small amounts. Normal α' precursors are made, but do not undergo carbohydrate processing, suggesting biosynthesis is blocked prior to the Golgi. β association appears to be required for processing and transport to the surface. The α chains are not expressed on the surface (severe deficiency) or in amounts which appear stoichiometrically limited by the small quantity of β produced (moderate deficiency). In addition to surface expression, patient granulocytes and monocytes lack the intracellular pool of Mac-1 and p150,95. After stimulation with fMet-Leu-Phe or phorbol ester, patient cells show little if any increase in Mac-1 and p150,95 surface expression [4,8].

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 leukocyte adhesion and

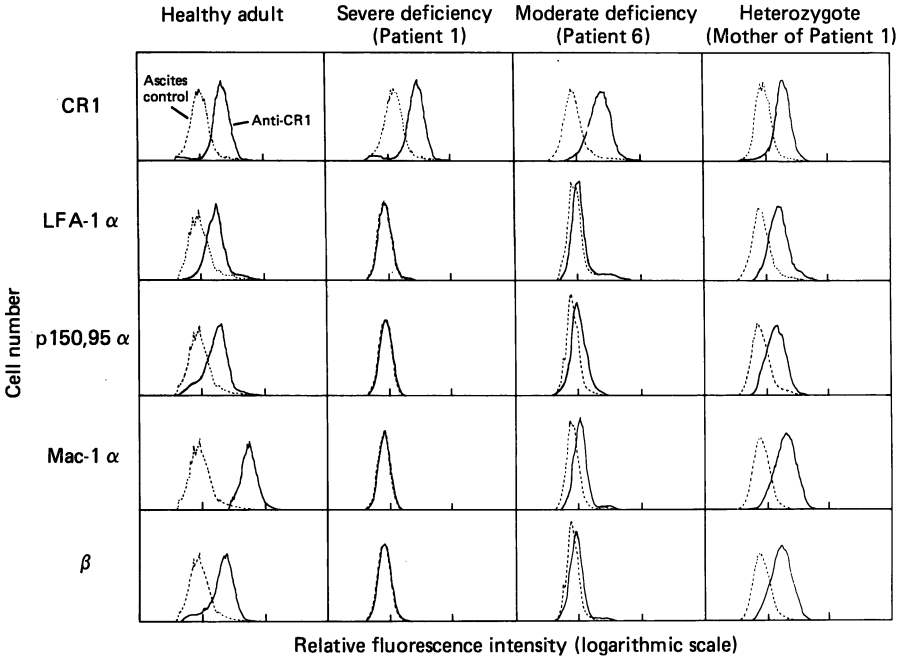

Fig. 2. *Immunofluorescence flow cytometry of granulocytes of representative severe and moderate deficiency patients, a heterozygote, and a healthy adult*

Unstimulated granulocytes were indirectly stained with antibodies to the CR1 or the indicated α or β subunits (solid lines) or control MAb (broken lines). A similar degree of deficiency was found if patient granulocytes were stained after fMet-Leu-Phe stimulation. [From Anderson *et al.* (1985) with permission.]

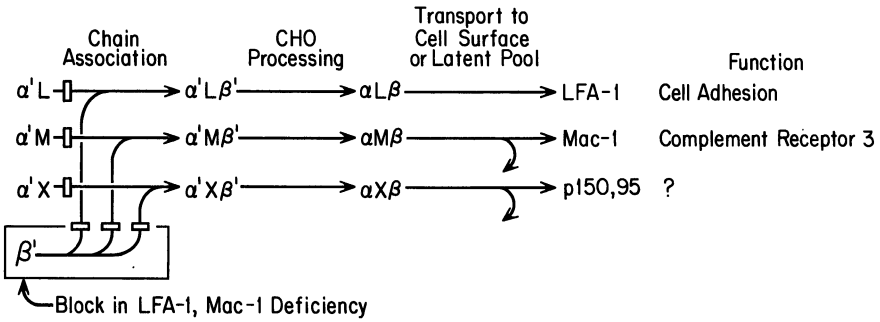

Fig. 3. *Biosynthesis of the Mac-1 , LFA-1 glycoprotein family* [4, 9]

Patient cells have a primary block in β subunit synthesis, and a secondary block in α subunit processing.

migration [8,11]. 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 [11,12]. Indeed, despite some initial controversy [13], it is clear that patients are deficient in the CR3 [11,14]. The increased surface expression of the CR3 induced by chemoattractants may be an important mechanism whereby granulocytes increase their ability to phagocytose opsonized

Table 2. *Assessments of adherence-dependent granulocyte functions*

Data presented with respect to each functional assay is represented by mean ± 1 S.D. value for each patient category derived from individual patient mean values of two to six separate experiments. Summarized from [8].

Functional assay	Severe* deficiency	Moderate† deficiency	Healthy adults
Chemotaxis			
fMet-Leu-Phe (10^{-8}M)	43±6‡	66±6	105±4
C5a	42±5	68±14	108±7
Adherence			
Baseline (saline)	12±2§	16±9	38±6
fMet-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‖	15±12	100±0
fMet-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¶	1.4±0.5	1.7±0.4
Oil Red O (iC3b)	1.9±1.2¶	2.4±1.2	7.0±3.1
C3-opsonized zymosan	4.6±0.7**	7.9±3.2	17.4±4.0

* Includes assessments on severe deficiency patients 1, 2 and 3.

† Includes assessments on moderate deficiency patients 4, 6, 7 and 8.

‡ Boyden assay values (mean ± 1 S.D.) for fMet-Leu-Phe or C5a (10% zymosan-activated serum) expressed as μm migration/40 min incubation.

§ Percent of granulocytes adhering to serum (6%) coated glass under baseline or stimulated conditions at 21 °C.

‖ Granulocyte aggregation responses to C5a (10% zymosan-activated plasma), fMet-Leu-Phe or phorbol myristate acetate (PMA), at 37 °C, measured by the increase in light transmittance and expressed as the % of the response to C5a.

¶ Dionylphthalate uptake (μg/10^6 granulocytes in 15 min).

** Slope of chemiluminescence evolution (10^{-5} × c.p.m.^{2})

micro-organisms as they migrate into inflammatory sites. However, the functional consequences of Mac-1, LFA-1 deficiency are much broader than for a complement receptor deficiency.

The recurrent soft tissue infections in patients appear due to an inability of granulocytes and monocytes to migrate into inflammatory sites [8,11]. There is an absence of pus formation and consequently, the common occurrence of necrotic, ulcerative, skin or mucous membrane infections. This has been confirmed by biopsies of infected tissues (gingiva and skin) and by Rebuck skin windows, which show profoundly impaired leukocyte mobilization. Following Rebuck skin abrasions, healthy control individuals show immigration of neutrophils at 2 and 4 h followed by monocytes at 6 h. Severely deficient patients show no mobilization of neutrophils or monocytes to the site even at 24 h, and leukocyte mobilization in moderately deficient patients is strikingly diminished and delayed. Thus, Mac-1, LFA-1 deficiency results in a profound defect in diapedesis, i.e., in the ability of leukocytes to leave the circulation by migrating between endothelial cells and through the basement membrane in order to accumulate at inflammatory sites.

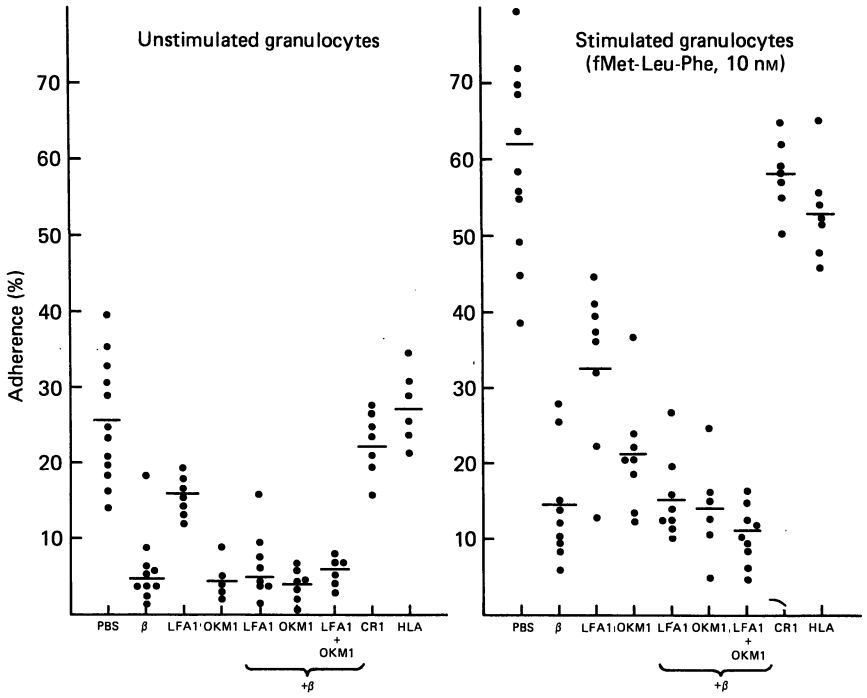

Fig. 4. *Effects of MAbs to the Mac-1, LFA-1 glycoprotein family on granulocyte adherence*

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 (phosphate-buffered saline) or stimulated (fMet-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 a F(ab′)$_2$ fragment of rabbit IgG directed against the human C3b receptor (anti-CR1) and a MAb against the HLA framework antigen (W6/32).

This dysfunction correlates with defects *in vitro* in adherence and adhesion-dependent functions, including chemotaxis, aggregation, orientation and phagocytosis of iC3b coated particles (Table 2) [8,11]. In additional patient studies, defects of granulocyte antibody-dependent cellular cytotoxicity, mononuclear cytotoxicity and cytolytic T-lymphocyte responses have been consistently demonstrated; in each assay, disturbed cytotoxic functions are related to diminished target cell binding. Observed abnormalities of lymphocyte proliferative responses to mitogens or allogeneic stimulation are presumably also related to impaired cell–cell interactions. With respect to many of these assays, the relative severity of functional abnormalities documented is directly related to the degree of molecular deficiency and severity of clinical infections or other manifestations in selected patients or kindreds.

In contrast to these characteristic abnormalities of adherence-dependent functions, adherence-independent functions of patient granulocytes or monocytes are uniformly normal. These include specific and saturable binding of the chemotactic factor, fMet-Leu-Phe, normal expression and function of receptors

for C5a, IgG Fc and CR1, cellular activation and shape change in suspension, oxidative metabolic and secretory (primary and secondary granule markers) responses to soluble stimulants, and normal assembly or function of cytoskeletal proteins [8].

Inhibition of Adherence-Dependent Functions by MAb

Binding of MAb to normal granulocytes largely reproduces the defects found in Mac-1, LFA-1 deficient patients. Both baseline and fMet-Leu-Phe-stimulated adherence are strikingly inhibited (Fig. 4). Spreading, chemotaxis, and aggregation are also inhibited. In most assays, the incorporation of MAbs during functional assessments are required to demonstrate effects or to promote the most effective inhibition of cell function. These observations suggest that a continual temporal expression of new Mac-1 binding sites contributes to these functions *in vitro* and presumably *in vivo*. The general order of potency of inhibition is anti-β > anti-(Mac-1 α) > anti-(p150,95 α) > anti-(LFA-1 α). 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 [10]. These effects are quite specific. They are obtained with $F(ab')_2$ fragments [anti-β and anti-(LFA-1 α)] and are not given by IgG MAb bound to other surface molecules (the CR1 and HLA-A,B). Functions not deficient in patient cells are not inhibited by these MAbs, i.e., shape change in suspension, fMet-Leu-Phe binding, superoxide generation, secondary granule secretion, and phagocytosis of Oil Red O (IgG).

A Dynamic Model of Chemotaxis and Diapedesis

The clinical and histopathological manifestations observed in Mac-1 deficient individuals as well as the specific molecular and functional deficits documented in patient cells suggest that many adherence-dependent inflammatory reactions of normal cells are mediated by the Mac-1, LFA-1 glycoprotein family, and specifically, that chemoattractant-stimulated hyperadherence and aggregation are mediated by an 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 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 'upregulation', leading to increased adherence of monocytes and granulocytes to endothelial substrates. We further propose that, in analogy with 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 sites (Fig. 5). Thus, we propose that the most important clinical manifestation of Mac-1 deficiency (i.e., the inability of granulocytes to migrate into inflammatory sites and form pus) is due to a lack of 'upregulation' 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

Table 3. *Effects of subunit-specific monoclonal antibodies to Mac-1 proteins on adherence-dependent and adherence-independent granulocyte functions*

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.

	Monoclonal antibody specificity					
	Mac-1 α					
Granulocyte function	(OKM1)	(LM 2/1.6)	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-fMet-Leu-Phe)	+	+	±	+	+	−
Spreading (glass)	+*	+*	−	±*	+*	−
Aggregation (C5a)	+	+	±	+	+	−
Chemotaxis (C5a)	+*		−	±*	+*	−
Phagocytosis (iC3b-Oil Red O)	+		−	−	+	−
Shape change (suspension)	−	−	−	−	−	−
fMet-Leu[^{3}H]Phe binding	−		−	−	−	−
Superoxide generation (PMA)	−	−	−	−	−	−
Secretion of glucuronidase, vitamin B_{12} transport protein (PMA)	−	−	−	−	−	−
Phagocytosis (IgG-Oil Red O)	−	−	−	−	−	−

* Requires presence of monoclonal antibody during assay for inhibitory effect.

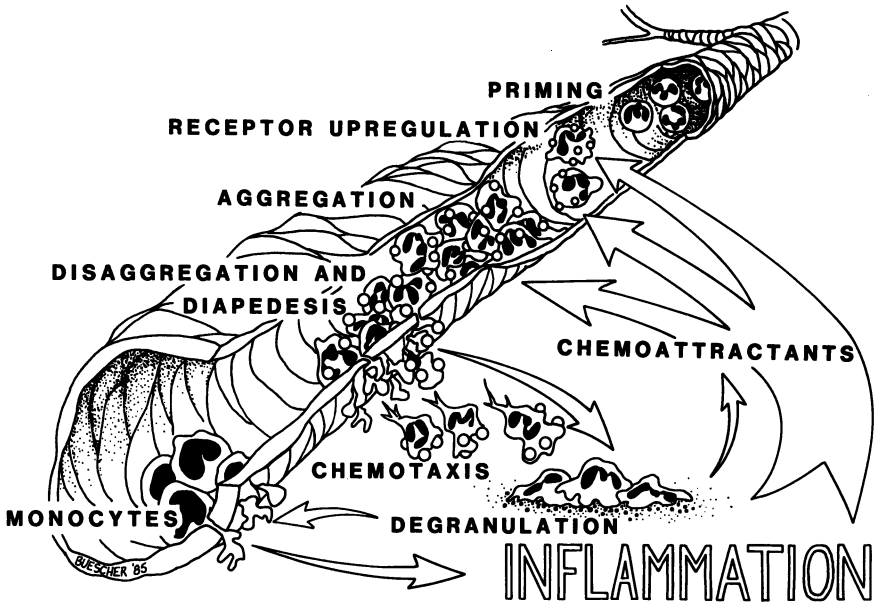

Fig. 5. *Chemoattractant-mediated Mac-1 and p150,95 upregulation, changes in leukocyte adherence, and diapedesis at inflammatory sites*

(Drawing by Dr. S. Buescher.)

adhesivity are not known, and further studies promise to provide important insights into the biology of inflammation. The Mac-1 α subunit gene has recently been cloned in this laboratory, and its sequence may soon yield important clues about structure and function. Whatever the mechanisms, the delivery of adhesive proteins to the cell surface in discrete packages, by fusion of specific granules or other secretory vesicles with the plasma membrane, allows an interesting speculation about the basis of chemotaxis. We propose that during granulocyte chemotaxis, 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 toward the chemoattractant. A focal point of high Mac-1 and p150,95 concentration would thus be formed in the plasma membrane at the site of fusion, and adhesion would be initiated or strengthened at this focal point. After further cell translocation, this focal point of attachment would near the uropod, and by this time, diffusion of the adhesion proteins in the plane of the membrane would have lowered their concentration and secondarily, the strength of adhesion. Because each granulocyte contains more than 1000 specific granules, this cycle would be repeated many times. This process could 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, thus allowing bulk membrane flow from the leading edge to the uropod, as has been hypothesized to effect cell locomotion [15].

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