

## SURFACE Mo1 (CD11b/CD18) GLYCOPROTEIN IS UP-MODULATED BY NEUTROPHILS RECRUITED TO SITES OF INFLAMMATION IN VIVO

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*Abstract*—Inasmuch as the recruitment of polymorphonuclear leukocytes (PMNs) to inflammatory foci in vivo involves adhesion-dependent events (e.g., margination, diapedesis, and directed migration), we sought to characterize the relationship between the local accumulation of PMNs in sterile peritonitis and their surface expression of the adhesion-promoting plasma membrane glycoprotein, Mo1 (CD11b/CD18). In an immunofluorescence analysis of PMNs isolated from rats injected intraperitoneally with sterile 1% glycogen solution, we detected a significant enhancement of surface Mo1 expression by exudative peritoneal PMNs. In contrast, no significant rise in Mo1 expression was noted over time by circulating intravascular PMNs (isolated simultaneously). However, these intravascular PMNs had the capacity to increase their surface Mo1 density upon exposure to peritoneal fluid supernatant at 37°C. These results demonstrate that PMNs at sites of inflammation in vivo do up-modulate their surface expression of the adhesion-promoting Mo1 glycoprotein during their recruitment from the circulating, intravascular leukocyte pool.

### INTRODUCTION

The recruitment of polymorphonuclear leukocytes (PMNs) from the intravascular compartment to extravascular sites of inflammation is a characteristic feature of the acute inflammatory response (reviewed in 1, 2). This accumulation of PMNs in vivo, stimulated by soluble chemotactic factors (1–3), appears to

be accompanied by several cellular activation events including degranulation (4, 5) and alterations in cellular responsiveness to stimulated superoxide production (5, 6), membrane depolarization (5), and chemotaxis (5, 7-9). When enhanced, these effects may in part be due to the up-modulation of surface FMLP receptor expression (5). Another PMN activation event of particular interest in the inflammatory response is the up-modulation of cell surface Mo1 receptor density. Mo1 (CD11b/CD18) is a leukocyte plasma membrane glycoprotein that promotes PMN adhesive functions, as demonstrated in vitro by the use of specific inhibitory murine anti-Mo1 monoclonal antibodies (reviewed in 10), and by the recognition of an inherited disorder in which PMNs from affected persons are deficient in Mo1 expression and exhibit markedly impaired cellular adhesive functions (reviewed in 10, 11). PMN surface Mo1 expression, in concert with various cellular adhesive properties, is enhanced in vitro in response to exposure to soluble activating factors, including FMLP (12, 13), C5a (14-16), LTB<sub>4</sub> (17, 18), adenine nucleotides (19), PMA, and calcium ionophore A23187 (12, 20, 21), and others. While it has been suggested that PMN Mo1 receptor up-modulation may occur in vivo (5, 14, 22-24), little is known about how this up-modulation might relate to the kinetics of PMN recruitment during inflammation.

Therefore, we undertook this study of an animal model of sterile peritonitis to more fully characterize the PMN response to localized acute inflammation, as manifested by the surface expression of the Mo1 glycoprotein. The results herein demonstrate that there is a rapid, significant, and sustained up-modulation of surface Mo1 glycoprotein, which accompanies the accumulation of PMNs at sites of inflammation in vivo, lending further support to the notion that PMNs undergo cellular activation during inflammatory recruitment.

## MATERIALS AND METHODS

*Reagents.* The following reagents were purchased from Sigma Chemical Company (St. Louis, Missouri): ammonium chloride (NH<sub>4</sub>Cl, A4514), disodium ethylene-diaminetetraacetate (EDTA, ED2SS), and glycogen type II (derived from oyster, G8751). Sodium bicarbonate (NaHCO<sub>3</sub>, 15421) was obtained from Columbus Chemical Industries, Inc. (Columbus, Wisconsin). Fluorescein isothiocyanate-conjugated goat anti-mouse IgG + IgM antibodies were obtained from Tago, Inc. (6253, Burlingame, CA).

*Solutions.* Phosphate-buffered saline (PBS, without calcium or magnesium) and Dulbecco's PBS (DPBS) were mixed according to standard formulations using reagent grade chemicals. NH<sub>4</sub>Cl erythrocyte lysis buffer consisted of NH<sub>4</sub>Cl 0.15 M, NaHCO<sub>3</sub> 0.01 M, and EDTA 0.001 M (adjusted to pH 7.20).

*Isolation of Peritoneal Elicited and Intravascular PMNs.* Pathogen-free, male, outbred Long-Evans rats weighing 400-450 g were purchased from the Charles River Company (Bar Harbor, Maine). For each experiment, five rats were injected intraperitoneally (under ether anesthesia)

with 30 ml of sterile 0.9% NaCl solution containing 1% (w/v) glycogen and then allowed to recover from anesthesia. Thereafter, at intervals of 1, 2, 3, 4, and 16 h following the injection, one rat was killed (by cardiac puncture under ether anesthesia), its peritoneal cavity opened, and the fluid therein collected and combined with two subsequent peritoneal washings using 0.9% NaCl solution. These elicited peritoneal cells were then pelleted, subjected to  $\text{NH}_4\text{Cl}$  erythrocyte lysis, washed, and resuspended at  $10 \times 10^6$  cells/ml in immunofluorescence wash buffer (21) at  $4^\circ\text{C}$  prior to staining with monoclonal reagents. Five milliliters of heparinized whole blood obtained from each rat at the time of cardiac puncture was subjected to  $\text{NH}_4\text{Cl}$  erythrocyte lysis (one part blood to eight parts  $\text{NH}_4\text{Cl}$  buffer at  $25^\circ\text{C}$  for 5–10 minutes). The leukocytes were then washed twice with PBS and suspended in DPBS prior to *in vitro* stimulation. Each experiment also included a control animal (anesthetized but not injected with glycogen) from which blood was obtained at time 0, as described above. Total and differential leukocyte counts were determined morphologically using Wright-Giemsa-stained intravascular and peritoneal specimens. Absolute PMN counts were calculated as the percentage of the total leukocyte count comprising segmented and band forms.

*Collection of Peritoneal Fluid Supernatant (PFS).* Two rats were injected intraperitoneally with sterile 0.9% NaCl solution containing 1% glycogen, as described above. After 4 h, approximately 15 ml of intraperitoneal fluid were collected from each rat and subjected to high-speed centrifugation to pellet suspended cells. Aliquots of the resulting cell-free PFS were stored at  $-80^\circ\text{C}$  until used for *in vitro* stimulation of isolated, intravascular PMNs.

*In Vitro Stimulation of PMNs.* Isolated leukocytes harvested at each time point were suspended at  $4\text{--}12 \times 10^6/\text{ml}$  in DPBS and placed in polypropylene culture tubes (Falcon Plastics, Oxnard, California). Cells were then exposed to an equal volume of undiluted PFS and incubated at  $37^\circ\text{C}$  for 30 min. Control cells received an equal volume of DPBS and were incubated for 30 min at either  $37^\circ\text{C}$  or  $4^\circ\text{C}$ . Cells were then diluted in ice-cold PBS, pelleted, and resuspended in immunofluorescence wash buffer (21) prior to further analysis.

*Immunofluorescence Analysis.* The generation and characterization of murine monoclonal antibodies anti-Mo1 (anti-CD11b, IgM; clone 17) (25) and anti-Mo2 (anti-CD14, IgM; clone 116) (26) have been described. Murine clone 17 anti-(human) Mo1 cross-reacts with the rat PMN homolog and inhibits rat PMN aggregation (Freyer and Todd, unpublished observations, August 1987). PMNs were subjected to indirect immunofluorescence staining for the expression of the Mo1 (CD11b) antigenic determinant relative to background staining by the isotype-identical negative-control antibody, anti-Mo2 (anti-CD14), and examined by flow cytometric analysis as described (21, 27). For experiments shown in Table 2, specific fluorescence intensity represents the computed mean channel number (0–230 channels, linear scale) of cells stained with the anti-Mo1 reagent minus the mean channel number of cells stained with anti-Mo2 reagent. In Table 2 below, numbers in parentheses indicate the “-fold” increase in specific fluorescence intensity of either PFS-stimulated intravascular PMNs or elicited peritoneal PMNs, as compared to unstimulated intravascular PMNs maintained at  $4^\circ\text{C}$ . The statistical significance of variations in the absolute PMN counts or in the stimulated increases in specific fluorescence intensity were evaluated by Student's *t* test.

## RESULTS

In examining neutrophil responses to localized inflammation in the rat, we first measured the number of PMNs recruited to the peritoneal cavity as a function of time, following the instillation of a local inflammatory agent [sterile 1% (w/v) glycogen solution]. As shown in Table 1, there was a profound rise in

**Table 1.** Peritoneal, Not Intravascular, PMN Counts are Rapidly Increased in Response to Localized Inflammation<sup>a</sup>

Time (hs)	Absolute PMN count <sup>b</sup>	
	Peritoneal (total)	Intravascular (per ml)
0		1.8 ± 1.3 (26.8 ± 9.5) <sup>c</sup>
1	2.1 ± 1.0 (34.3 ± 28.9) <sup>c</sup>	0.8 ± 0.3 <sup>d</sup> (12.0 ± 0.8) <sup>c</sup>
2	12.2 ± 9.1 (75.4 ± 21.6)	2.8 ± 1.1 (31.0 ± 8.6)
3	50.9 ± 17.1 (98.2 ± 2.0)	2.7 ± 0.8 (34.2 ± 10.8)
4	119.3 ± 80.8 (98.4 ± 2.1)	2.1 ± 1.2 (30.0 ± 9.7)
16	212.0 ± 76.3 (90.2 ± 15.0) <sup>c</sup>	9.5 ± 7.2 <sup>d</sup> (63.7 ± 28.7) <sup>c</sup>

<sup>a</sup>For each experiment ( $N = 5$ ), five rats were injected intraperitoneally with 30 ml sterile 1% glycogen solution. Thereafter, at 1, 2, 3, 4, and 16 h following the injection, one rat was killed for the collection of accumulated peritoneal and intravascular PMNs, as described in Materials and Methods. Each experiment also included a control animal (not injected with glycogen) from which blood was similarly obtained at time 0. For all peritoneal and intravascular specimens, total leukocyte counts were determined on cell suspensions examined in a standard hemacytometer, and differential leukocyte counts were determined morphologically using Wright-Giemsa stained slide preparations.

<sup>b</sup>The absolute PMN count (calculated as the percentage of the total leukocyte count comprising segmented and band forms) of peritoneal and intravascular specimens was determined for each time point. Peritoneal values are expressed as the mean total absolute PMN count  $\times 10^6 \pm SD$ , and intravascular values as the mean absolute PMN count  $\times 10^6$  per milliliter of whole blood  $\pm SD$ . For both, numbers in parentheses represent the mean percentage of PMNs  $\pm SD$ .

<sup>c</sup> $N = 4$ .

<sup>d</sup> $P = NS$  (as compared to intravascular PMN count at time 0).

<sup>e</sup> $N = 3$ .

the mean absolute peritoneal PMN count that was directly related to the duration of the inflammatory exposure. Whereas in normal rats there were negligible numbers of resident peritoneal PMNs (time 0), after 1 and 16 h there were mean total peritoneal PMN counts of 2.1 and  $212.0 \times 10^6$ , respectively, signifying a 100-fold increase over this time interval. In contrast, there was no significant variation over time in the mean intravascular PMN count. Additionally, as shown in Table 1, we observed that within 2–3 h of intraperitoneal glycogen injection, over 90% of the accumulated leukocytes were PMNs, whereas the percentage of intravascular PMNs did not vary appreciably over time. These results indicate that in this *in vivo* model of localized (intraperitoneal) inflammation, there was a rapid, marked, and reproducible accumulation of PMNs that was not accompanied by changes in the PMN count of the circulating, intravascular leukocyte pool.

To assess the relative state of activation of rat PMNs in the intravascular and peritoneal compartments, we examined the surface expression of the CD11b/CD18 (Mo1) glycoprotein, which is an adhesion-promoting molecule

whose density on the PMN plasma membrane is up-modulated by exposure to inflammatory factors (10) and may play a role in the diapedesis and directed migration of PMNs responding to an inflammatory stimulus. In the immunofluorescence analysis shown in Table 2, surface Mo1 expression by isolated peritoneal PMNs was significantly increased as compared to intravascular PMNs kept unstimulated at 4°C. This increase in specific fluorescence intensity (SFI) above that of unstimulated intravascular PMNs isolated simultaneously ranged from 4.3- to 8.6-fold over the first 4 h ( $P = \text{NS}$ ,  $<0.01$ ,  $<0.01$ , and  $<0.05$ , for 1, 2, 3, and 4 h, respectively) and was of lesser magnitude after 16 h. We detected no significant variation over time in the "baseline" SFI of unstimulated intravascular PMNs. However, as shown in Table 2, these isolated intra-

**Table 2.** Increased Surface Mo1 Expression is Detected on PMNs Isolated from Inflammatory Focus or Stimulated In Vitro<sup>a</sup>

Time (hs)	Specific fluorescence intensity <sup>b</sup>		
	Intravascular PMNs		Peritoneal PMNs
	4°C	PFS-stimulated	4°C
0	1.8 ± 1.3	8.9 ± 5.6 <sup>c</sup> (4.9)	
1	3.4 ± 1.8	16.6 ± 7.7 <sup>d</sup> (4.9)	14.5 ± 11.4 <sup>e</sup> (4.3)
2	1.9 ± 1.2	8.6 ± 5.4 <sup>f</sup> (4.5)	13.1 ± 6.4 <sup>d</sup> (4.5)
3	2.3 ± 1.7	12.1 ± 6.9 <sup>f</sup> (5.3)	12.1 ± 5.0 <sup>d</sup> (5.3)
4	1.6 ± 1.1	10.3 ± 4.9 <sup>d</sup> (6.4)	13.8 ± 9.2 <sup>f</sup> (8.6)
16	5.5 ± 5.1 <sup>g</sup>	15.0 ± 10.4 (2.7)	8.6 ± 5.3 (1.6)

<sup>a</sup>Peritoneal and intravascular PMNs ( $N = 5$ ) were collected as described in Table 1, footnote *a*. Isolated peritoneal PMNs were maintained at 4°C, whereas isolated intravascular PMNs were either kept at 4°C or exposed to cell-free peritoneal fluid supernatant (PFS, prepared in Materials and Methods) at 37°C for 30 min. PMNs were then stained by indirect immunofluorescence for the expression of the Mo1 (CD11b) determinant, relative to the background staining by an isotype-identical negative-control antibody.

<sup>b</sup>Values represent the mean specific fluorescence intensity ± *SD* (0–230 channels, linear scale) of cells stained by anti-Mo1 antibody; nonspecific background staining by the negative-control antibody has been subtracted in each case. Photomultiplier tube setting was 1000 mV in all experiments. Numbers in parentheses represent the -fold increase of specific fluorescence intensity of either peritoneal PMNs (4°C) or intravascular PMNs exposed to PFS at 37°C for 30 min, above that of intravascular PMNs maintained at 4°C. The corresponding relative increase in surface Mo1 expression stimulated by warming of intravascular PMNs in the absence of PFS was 2.7-, 2.4-, 3.4-, 3.1-, 2.6-, and 2.3-fold at 0, 1, 2, 3, 4, and 16 h, respectively.

<sup>c</sup> $P = 0.05$ .

<sup>d</sup> $P < 0.01$ .

<sup>e</sup> $P = \text{NS}$ .

<sup>f</sup> $P < 0.05$ .

<sup>g</sup>While there was a tendency for intravascular PMNs isolated at 16 h to exhibit higher than baseline (time 0) surface Mo1 expression (which could indicate delayed intravascular PMN activation), the difference did not reach statistical significance ( $P = \text{NS}$ ).

vascular PMNs, when exposed to peritoneal fluid supernatant (PFS) *in vitro* at 37°C for 30 min, could up-modulate their surface Mo1 antigen density to levels that approached or exceeded those of peritoneal PMNs isolated simultaneously. This stimulated enhancement of Mo1 expression by PFS-exposed intravascular PMNs averaged 5.2-fold above that of circulating PMNs kept at 4°C (significant 0–4 h), with a lesser increase stimulated after 16 h. Isolated intravascular PMNs incubated in buffer alone at 37°C for 30 min (in the absence of PFS) exhibited an intermediate enhancement of Mo1 expression (Table 2, footnote *b*). Collectively, these results demonstrate that an increase in the surface expression of the Mo1 adhesion glycoprotein is a feature characteristic of PMNs recruited from the intravascular leukocyte pool to sites of acute inflammation.

## DISCUSSION

Since the accumulation of PMNs at sites of inflammation *in vivo* is dependent upon several adhesion-related processes (e.g., margination, diapedesis, and directed migration), in this study we sought to characterize the relationship between the inflammatory recruitment of PMNs and their surface expression of the adhesion-promoting plasma membrane glycoprotein, Mo1. Mo1 (CD11b/CD18; CR3; gp 155,95) is a member of the CD11/CD18 family of structurally related heterodimers expressed on the surfaces of PMNs and certain mononuclear phagocytes (10) and has been shown to promote multiple cellular adhesive interactions, including PMN adherence and spreading on artificial (25, 28) and cellular (29, 30) substrates, aggregation (14, 28, 29), chemotaxis (25, 28), and binding of C3bi-opsonized particles (31–33) and gram-negative bacteria (34). The Mo1 glycoprotein exists in an intracellular pool associated with secondary and/or tertiary granules (16, 20, 35) and is rapidly translocated to the plasma membrane upon exposure of the cell to a variety of activating factors [e.g., FMLP (12, 13), C5a (14–16), LTB<sub>4</sub> (17, 18), ATP (19), PMA and A23187 (12, 20, 21), and others], thus serving as an “activation marker” for PMNs.

We have found that PMNs accumulating at sites of acute inflammation *in vivo* do indeed significantly up-modulate their surface density of the Mo1 antigen, as compared to intravascular PMNs isolated simultaneously from the same animal. Moreover, we have shown that this enhancement occurs rapidly (within 1 h of recruitment), is sustained with further local recruitment of PMNs, and is not accompanied by significant elevations of either the Mo1 expression or the total count of intravascular PMNs. Thus, our findings extend the observations of Zimmerli et al. (5), who reported a twofold increase in surface Mo1 expression by exudative guinea pig and human PMNs analyzed at a single time point (14 h) following the introduction of an inflammatory stimulus.

Since Mo1 up-modulation coincided with PMN accumulation at the site of peritoneal inflammation, we evaluated the possibility that the administration of specific anti-Mo1 monoclonal antibody might interfere with this process. However, when a similar group of rats were pretreated (1 mg intravenously 1 h before glycogen injection) with a murine anti-human Mo1 antibody (clone 17 producing an IgM antibody cross-reacting with the rat homolog), there was no significant reduction in intraperitoneal PMN accumulation 4 h after glycogen injection (despite the presence of saturating concentrations of anti-Mo1 antibody in the plasma and peritoneal fluid), as compared to negative control rats receiving no antibody ( $113 \pm 10$  vs.  $106 \pm 5 \times 10^6$ , respectively; mean total peritoneal PMN count  $\pm$  SD,  $N = 3$ ). This is in contrast to the findings of Rosen and Gordon (36), who recently reported a 75% inhibition of myelomonocytic cell accumulation in a murine model of sterile peritonitis where mice were pretreated with an anti-Mo1 antibody. The discrepancy in these results is most likely due to a difference in epitope recognition in which one antibody but not the other competitively binds to a region of the Mo1 glycoprotein that promotes PMN adhesive interactions critical to inflammatory recruitment in vivo (25, 28, 29). The importance of the CD11/CD18 family of adhesion glycoproteins in PMN migration to inflammatory foci has been indicated by the inhibitory effects of other CD11/CD18-specific monoclonal reagents in several additional animal models of acute inflammation (37-41).

While some degree of surface Mo1 expression is required for normal PMN adhesive function in the inflammatory response, the functional importance of the increase in receptor density above baseline exhibited by activated cells is unclear. The physiologic significance of enhanced Mo1 expression is suggested by its detection in the settings of clinically active systemic lupus erythematosus (22), hemodialysis leukopenia (14), severe burns (23), and the treatment of cancer patients with intravenous GM-CSF (24). However, a series of recent observations suggest that qualitative, rather than quantitative, changes in existing plasma membrane Mo1 receptors could underlie the enhanced cellular adhesiveness observed in stimulated PMNs. Wright and coworkers reported the activation and deactivation of CR3 (Mo1) binding activity in human PMNs and monocytes without a corresponding change in receptor number (42, 43). More recently, Vedder and Harlan described increased adherence of human PMNs to cultured endothelial cell monolayers that was independent of the enhancement of CD11b or CD18 determinants detected on PMNs stimulated by PMA, A23187, or FMLP (44). In addition, Buyon et al. reported that maximal FMLP-stimulated aggregation of human PMNs did not correspond to increases in surface Mo1 expression (45). Examples of qualitative changes in the Mo1 receptor that might confer functional responsiveness include topographic clustering of CR3 receptors on the plasma membrane (46, 47) or glycoprotein phosphorylation (43, 48). Thus, the well-documented inhibitory effect of certain anti-Mo1

monoclonal reagents on the recruitment of PMNs to inflammatory foci in vivo (36-41) could be due to an interference with such qualitative changes. If true, the enhancement of Mo1 expression by PMNs recruited during inflammation, as reported here, may serve a purpose yet to be elucidated.

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