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### Research Article

The iC3b receptor (CR3) is required for neutrophil adhesive functions, including homotypic aggregation. Because stimuli that enhance neutrophil adhesion also induce up-regulation of surface CR3, it is widely held that these two responses are causally related. We have dissociated CR3 display (immunofluorescence) from CR3 function (aggregation). Neutrophils isolated at 4 degrees C and rewarmed to 37 degrees C up-regulated surface CR3 twofold, but did not aggregate. The kinetics of FMLP-induced CR3 up-regulation were discordant with those of aggregation. In the absence of extracellular divalent cations, CR3 expression increased twofold after exposure to FMLP, but neutrophils did not aggregate. FMLP elicited 3.5-fold more aggregation than the ionophore A23187, yet less than one-half as much CR3 up-regulation. 3 mM sodium salicylate inhibited aggregation 55 +/- 4%, but had no effect on CR3 up-regulation. Conversely, 1 mM tetracaine completely inhibited CR3 up-regulation, while significantly enhancing aggregation. Neutroplasts expressed CR3, but did not up-regulate the receptor; in contrast, FMLP induced CR3-dependent aggregation of neutroplasts. We conclude that, although constitutive surface CR3 is required for neutrophil aggregation, the up-regulation of CR3 is neither necessary nor sufficient to promote cell-cell adhesion.

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# Up-regulation of the iC3b Receptor (CR3) Is Neither Necessary Nor Sufficient to Promote Neutrophil Aggregation

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## Abstract

The iC3b receptor (CR3) is required for neutrophil adhesive functions, including homotypic aggregation. Because stimuli that enhance neutrophil adhesion also induce up-regulation of surface CR3, it is widely held that these two responses are causally related. We have dissociated CR3 display (immunofluorescence) from CR3 function (aggregation). Neutrophils isolated at 4°C and rewarmed to 37°C up-regulated surface CR3 twofold, but did not aggregate. The kinetics of FMLP-induced CR3 up-regulation were discordant with those of aggregation. In the absence of extracellular divalent cations, CR3 expression increased twofold after exposure to FMLP, but neutrophils did not aggregate. FMLP elicited 3.5-fold more aggregation than the ionophore A23187, yet less than one-half as much CR3 up-regulation. 3 mM sodium salicylate inhibited aggregation 55±4%, but had no effect on CR3 up-regulation. Conversely, 1 mM tetracaine completely inhibited CR3 up-regulation, while significantly enhancing aggregation. Neutroplasts expressed CR3, but did not up-regulate the receptor; in contrast, FMLP induced CR3-dependent aggregation of neutroplasts. We conclude that, although constitutive surface CR3 is required for neutrophil aggregation, the up-regulation of CR3 is neither necessary nor sufficient to promote cell-cell adhesion.

## Introduction

In the course of inflammation, neutrophils adhere to endothelium and to each other (1–3). Adhesion is mediated by a group of related cell-surface glycoproteins that includes the iC3b receptor (CR3)<sup>1</sup> (1, 4). Although first recognized by rosette assays as a receptor on myeloid cells with relative specificity for iC3b (5), CR3 was subsequently shown to be identical to a heterodimeric glycoprotein recognized by the MAbs Mo-1 and Mac-1, and sometimes referred to as the Mo-1 or Mac-1 antigen (4, 6). CR3 consists of a 165-kD alpha chain (CD11b) noncovalently linked to a 95-kD beta chain (CD18) (7). The beta chain is shared by two other leukocyte surface glycopro-

teins, LFA-1 and gp150/95, which together form a functionally important family of membrane glycoproteins designated the LFA-1 family (4). The expression of these three molecules varies according to lineage and stage of maturation of hematopoietic cells (8). All three molecules have been implicated in various functions of leukocytes related to adhesion (1, 9). CR3, the predominant species on mature granulocytes, is also present on monocytes, macrophages, and null cells (5, 10).

Several lines of evidence suggest that CR3 is a major adhesion-related molecule of human neutrophils. A recently recognized group of pediatric patients, genetically deficient in all three LFA-1 family molecules, suffer from recurrent soft tissue infections (1, 11, 12). Neutrophils from these patients exhibit defects in such adhesion-related functions as aggregation, spreading on surfaces, directed migration, and attachment to endothelial cells (12–15). Normal human neutrophils, treated in vitro with a subset of available anti-CR3 MAbs, exhibit defects indistinguishable from those of neutrophils from CR3-deficient patients (9, 12, 16–18).

The number of CR3 molecules on resting neutrophils is reported to be 10,000–20,000 per cell, although this number depends on isolation procedures (5, 19). After exposure to stimuli such as con A, FMLP, zymosan-activated serum (ZAS), phorbol myristate acetate (PMA), or calcium ionophores, neutrophils augment their surface expression of CR3 5- to 10-fold, a process often referred to as up-regulation (5, 11, 19–23). CR3 up-regulation is rapid and is not dependent on protein synthesis (19, 22, 23). Studies indicate that up-regulation results from the translocation of preformed receptors to the plasma membrane from an intracellular source that cosediments with the specific granule fraction of neutrophil lysates (23, 24).

Neutrophils exposed to stimuli that enhance cell-cell adhesiveness also display more CR3 on their surface. Because of this association, it is widely held that up-regulation of CR3 and augmented adhesiveness are causally related (8, 9, 12, 14, 22, 23). In this study, we examined the relationship between the up-regulation of CR3 on the human neutrophil and its functional activation, i.e., its capacity to promote cell-cell aggregation. We investigated these two responses with respect to their kinetics, calcium requirements, pharmacologic sensitivities, and dependence on various stimuli. We have also studied the same two responses in neutrophilic cytoplasts that lack specific granules. Surprisingly, our results demonstrate that the increased surface expression of CR3 that accompanies neutrophil activation is neither necessary nor sufficient to induce neutrophil aggregation.

## Methods

**Reagents.** PBS contained 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, and 8 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.45. Hepes buffer contained 149 mM NaCl, 5 mM KOH, 1.3 mM CaCl<sub>2</sub>, 1.2 mM MgCl<sub>2</sub>, and 10 mM

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1. Abbreviations used in this paper: CR3, iC3b receptor; GAM-FITC, fluorescein-conjugated goat anti-human immunoglobulin; RFU, relative fluorescence unit; ZAS, zymosan-activated serum.

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Hepes, pH 7.45. The above constituents as well as DMSO, Ficoll 70, paraformaldehyde, sodium salicylate, and tetracaine were purchased from Sigma Chemical Co. (St. Louis, MO). A23187 and PMA were purchased from Calbiochem-Behring Corp. (La Jolla, CA). Cytochalasin B was obtained from the Aldrich Chemical Co. (Milwaukee, WI). FMLP was purchased from Vega Biotechnologies, Inc. (Tucson, AZ). ZAS was prepared by incubating human sera with 10 mg/ml zymosan at 37°C for 30 min (ICN Pharmaceuticals, Cleveland, OH) followed by centrifugation. Leukotriene B<sub>4</sub> (LTB<sub>4</sub>) containing < 7% (5*S*,12*S*)-diHETE was prepared as previously reported (25).

**Neutrophil isolation.** Blood was collected from normal volunteers, anticoagulated with 10 mM Na<sub>2</sub>EDTA, and immediately placed on ice. All subsequent isolation steps were performed at 0–4°C. Neutrophils were isolated by means of Ficoll/Hypaque gradient centrifugation followed by dextran sedimentation and hypotonic lysis as previously described (26). Neutrophils were washed and maintained in ice-cold PBS.

**Neutroplast preparation.** Neutroplasts were prepared by the method of Roos et al. (27). Briefly, 10<sup>8</sup> neutrophils were mixed with 12% (wt/vol) Ficoll 70 containing 5 μg/ml cytochalasin B and layered over a discontinuous gradient of 16 and 25% Ficoll 70 prewarmed to 37°C containing 5 μg/ml cytochalasin B. The cells were spun in an L-2 ultracentrifuge with a SW27 rotor (Beckman Instruments, Inc., Fullerton, CA [82,000 g, 35°C, 30 min]). Neutroplasts were harvested from the layer that formed at the 12/16% Ficoll interface. These were washed three times with ice-cold Hepes and maintained on ice. Light microscopic counts confirmed that on average, one neutroplast was formed from each neutrophil loaded onto the gradient.

**Antibodies.** The murine MAb MN-41, an IgG<sub>1</sub> directed to the human CR3 alpha chain (CD11b) (10), was the generous gift of Dr. Gordon Ross (Division of Rheumatology and Immunology, University of North Carolina School of Medicine, Chapel Hill, NC). Murine MAb CLB-LFA-1-1, the kind gift of Dr. Frank Miedema (Central Lab of the Netherlands, Red Cross Blood Transfusion Service, Amsterdam, The Netherlands), is an IgG<sub>1</sub> directed to the common LFA-1/Mac-1/gp165/95 beta chain (CD18) (28). MOPC-21 is a murine IgG<sub>1</sub> MAb of unknown specificity and was used as an isotype control. Murine hybridoma C-40 was developed in our laboratory against human chronic myelogenous leukemia cells and produces a IgM MAb directed to a determinant on human neutrophils unrelated to CR3 (unpublished data). Mouse ascites was used as the source of MAbs. The immunoglobulin content of the various ascites was 1–5 mg/ml. Whole, purified polyclonal fluorescein-conjugated goat anti-mouse immunoglobulin (GAM-FITC; Coulter Electronics, Inc., Hialeah, FL) was used as the second stage antibody in indirect immunofluorescence staining. Qualitatively identical results were obtained when fluorescein-conjugated affinity purified F(ab')<sub>2</sub> fragments of goat anti-mouse IgG (Cappel Laboratories, Cochranville, PA) were substituted for the GAM-FITC. Results were confirmed by direct immunofluorescent analysis using fluoresceinated purified MN-41, which was prepared by standard procedures (29).

**Immunofluorescent analysis.** 10<sup>6</sup> neutrophils or 3 × 10<sup>6</sup> neutroplasts were resuspended in 1 ml Hepes buffer or, in selected experiments, divalent cation-free PBS. Control cells were kept on ice. Duplicate samples were warmed to 37°C for 10 min before the addition of stimuli for varying times. In certain experiments, cells were warmed in buffer containing 3 mM sodium salicylate or 1 mM tetracaine. After exposure to stimulus (or buffer control), reactions were stopped by the addition of 2 vol of ice-cold buffer. In kinetic experiments, the reaction was stopped with 2 vol of ice-cold paraformaldehyde (1% wt/vol) in Hepes. After a 5-min incubation, the fixative was removed by washing before the staining process. Cells were washed and the pellets were resuspended in 0.1 ml Hepes buffer containing MAb at a 1:400 dilution of ascites fluid (found in preliminary experiments to exceed the saturating concentration for MN41 staining of CR3) for 20 min on ice. Background staining with control MAb was not affected by increasing or decreasing 10-fold the concentration of MOPC-21 ascites. Then the cells were washed three times and resuspended in 0.2 ml Hepes buffer

containing a saturating concentration of GAM-FITC for 20 min on ice. After three final washes the cells were fixed with paraformaldehyde (1% wt/vol) and refrigerated before cytofluorographic analysis. Microscopic examination of stained neutrophils confirmed that cell clumps were disrupted by the washing procedure (> 95% of cells were singlets) before cytofluorographic analysis. Cells were analyzed with an Ortho 50H cytofluorograph interfaced with a Ortho 2150 computer (Ortho Diagnostic Systems, Inc., Raritan, NJ) run in the linear mode with the arithmetic mean channel of fluorescence distribution automatically computed. The mean fluorescence channel of unstimulated cells continually held at 0–4°C was arbitrarily assigned a relative fluorescence unit (RFU) of 100 and the other samples were normalized accordingly.

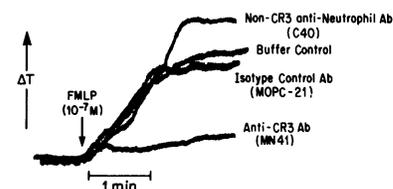
**Aggregation.** 1.25 × 10<sup>6</sup> neutrophils or 3.75 × 10<sup>6</sup> neutroplasts were resuspended in Hepes buffer or 0.1 ml PBS in the presence or absence of 3 mM sodium salicylate or 1 mM tetracaine and warmed to 37°C for 10 min in a siliconized cuvette. Aggregation was measured as the change in the transmittance of light through the stirred (900 rpm) suspensions of cells in a dual chamber aggregometer (Payton Scientific, Inc., Buffalo, NY). Responses were compared by calculating the areas under the aggregation curves in the first minute using a Micro Plan II morphometric tablet (Laboratory Computer Systems, Inc., Cambridge, MA). Aggregometry was corroborated with manual cell/clump counts in the light microscope.

**Statistics.** Significant differences were determined by the *t* test for paired data.

## Results

**Effect of MAb MN-41 on neutrophil aggregation.** Since some but not all MAbs directed to the alpha chain of CR3 inhibit neutrophil adhesion, we tested the ability of MN-41 to inhibit cell-cell aggregation. As shown in Fig. 1, incubation of neutrophils with MN-41 (1:400) markedly inhibited FMLP-induced aggregation. Ascites containing murine MAb CLB-LFA-1-1 directed against the CR3 beta chain gave similar inhibition (not shown). No inhibition was seen with identical concentrations of either MOPC-21, an irrelevant isotype control MAb, or C40, a murine anti-human neutrophil MAb not reactive with CR3. MN-41 also effectively inhibited neutrophil aggregation in response to the ligands C5a (present in ZAS) and LTB<sub>4</sub>, the calcium ionophores ionomycin, and A23187 and PMA, a direct activator of protein kinase C (Table I).

**Effect of temperature and FMLP on CR3 expression.** The warming of isolated neutrophils from 4 to 37°C increased the surface expression of CR3, as measured by MN-41 (anti-CD11b), to 168 ± 7% of control (*P* < 0.00001) by 11 min (Fig. 2). A similar increase (147 ± 7%, *P* < 0.01) was observed when CLB-LFA-1-1 (anti-CD18) was substituted for MN-41. The continued warming of cells over 40 min further increased CR3 expression to 205 ± 10% of control (*P* < 0.0005 versus cells



**Figure 1.** The effect of MN-41 on FMLP-induced neutrophil aggregation (curves representative of seven experiments). Neutrophils were preincubated in Hepes buffer with or

without various MAbs (1:400 vol/vol). Aggregation was measured as described in Methods after the addition of 10<sup>-7</sup> M FMLP. MN-41 is a murine IgG<sub>1</sub> MAb directed to the alpha chain of human CR3. MOPC-21 is a murine IgG<sub>1</sub> MAb with undetermined specificity. C40 is a murine IgM MAb directed to a human neutrophil surface antigen distinct from CR3.

**Table I. Effect of Mab MN-41 on Neutrophil Aggregation Induced by Various Agents**

Agent	n	Aggregation*	
		+MOPC-21 <sup>‡</sup>	+MN-41 <sup>‡</sup>
PMA (1 µg/ml)	(7)	87±11	6±1 <sup>§</sup>
FMLP (100 nM)	(7)	128±14 <sup>  </sup>	31±6 <sup>§</sup>
ZAS (10% vol/vol)	(4)	96±16	42±12 <sup>  </sup>
LTB <sub>4</sub> (200 nM)	(4)	97±15	44±10 <sup>†</sup>
Ionomycin (200 nM)	(4)	85±5	24±6 <sup>†</sup>
A23187 (10 µM)	(4)	83±13	41±14 <sup>  </sup>

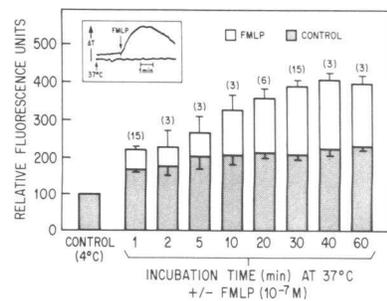
\* Aggregation was measured as described in Methods and quantitated by taking the area (in squared centimeters) under each curve in the first 2 min after addition of the agent. Data are expressed as the percent control aggregation in buffer alone (mean±SEM).

<sup>‡</sup> Ascites (1:400 vol/vol) containing MABs was added to neutrophil suspensions 1 min before the addition of the agent. MN-41 is directed to the α-chain of CR3; MOPC-21 is an isotype control MAB.

<sup>§</sup> P < 0.0001, <sup>||</sup> P < 0.05, <sup>†</sup> P < 0.01 versus buffer control.

warmed for 11 min). The insert in Fig. 2 shows the aggregation curves of cells warmed to 37°C with or without the subsequent addition of FMLP. Despite a doubling of surface CR3, neutrophils warmed from 0 to 37°C, but not exposed to a stimulus such as FMLP, do not aggregate.

10<sup>-7</sup> M FMLP had no significant effect on the CR3 expression of neutrophils maintained at 0°C. CR3 expression on neutrophils warmed to 37°C for 10 min and then exposed to 10<sup>-7</sup> M FMLP for increasing amounts of time is also shown in Fig. 2. After 1 min of FMLP exposure CR3 expression increased from 168±7 to 221±8 RFU (P < 0.0001). Therefore, 57±4% of the increase in CR3 expression seen after a 1-min exposure to FMLP was background attributable to warming alone. Not until 10 min of FMLP exposure did the increment in CR3 expression induced by the chemoattractant exceed that caused by warming alone. Moreover, after cells had been warmed in the absence of chemoattractant for 50 min, CR3 expression (223±18 RFU) was not different from that on cells warmed for 10 min and then exposed to FMLP for 1 min (221±8 RFU). Despite the display of an equal number of CR3 molecules on the cell surface, the cells warmed for 50 min in the absence of chemoattractant did not aggregate.



**Figure 2.** The effect of temperature and FMLP on neutrophil CR3 expression and aggregation. Neutrophils isolated at 4°C were warmed to 37°C for 10 min in Hapes buffer and then incubated in the presence or absence of 10<sup>-7</sup> M FMLP for the times indicated

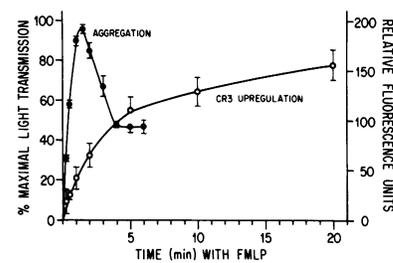
below each bar. The cells were then stained and subjected to cytofluorographic analysis as described in Methods. The results (RFU) are expressed as the mean±SEM of the number of experiments indicated in parentheses. The inset shows representative aggregation curves of similarly treated cells.

**Kinetics of CR3 up-regulation and neutrophil aggregation.**

Based on these results, we defined FMLP-attributable CR3 up-regulation as the increment (RFU) in CR3 expression on cells exposed to FMLP above the expression elicited by warming alone. We then compared the kinetics of FMLP-attributable up-regulation with those of neutrophil aggregation. As shown in Fig. 3, neutrophils aggregated within seconds of exposure to 10<sup>-7</sup> M FMLP. Aggregation was maximal at 90 s and was followed by disaggregation, despite the continued presence of FMLP. Disaggregation was confirmed by cell/clump counts in the light microscope as described by Hoffstein et al. (30). The kinetics of CR3 up-regulation were discordant with those of aggregation (Fig. 3). At the peak of aggregation (1 min), CR3 up-regulation was only 26±7% maximal. Moreover, aggregation reversed after 90 s, whereas CR3 up-regulation continued for more than 20 min.

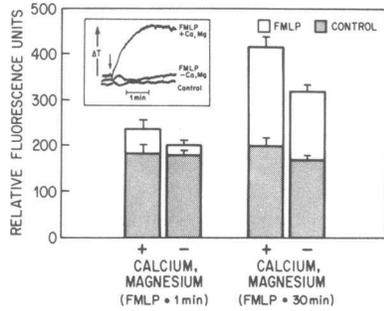
**Divalent cation requirements.** To analyze the discordance in the kinetics of these two processes, we next examined the divalent cation requirements of neutrophil CR3 up-regulation and aggregation. Fig. 4 compares CR3 expression on neutrophils isolated from blood anticoagulated with 10 mM EDTA and then maintained in calcium- and magnesium-free buffer with that on cells maintained in calcium- and magnesium-replete buffer. CR3 expression after exposure to 10<sup>-7</sup> M FMLP was significantly diminished in the absence of extracellular divalent cations both at 1 and 30 min. This inhibition, however, was not complete: in the absence of extracellular calcium and magnesium, exposure of neutrophils to 10<sup>-7</sup> M FMLP resulted in a small but significant up-regulation of surface CR3 at 1 min (177±9 vs. 200±9 RFU, P < 0.05 control vs. FMLP) and a twofold increase at 30 min (168±9 vs. 320±12 RFU, P < 0.001 control vs. FMLP). In comparison, the inset in Fig. 4 shows that neutrophils did not aggregate in the absence of extracellular divalent cations.

**Stimulus dependence.** An examination of responses to a 1-min exposure of neutrophils to diverse stimuli indicated that the extent of aggregation was not related to the magnitude of CR3 up-regulation (Table II). 10<sup>-5</sup> M of the calcium ionophore A23187 elicited twofold greater CR3 up-regulation than the ligand FMLP (10<sup>-7</sup> M). In comparison, FMLP elicited 3.5-fold more aggregation than A23187. This difference was not a function of comparing an ionophore to a ligand since, of



**Figure 3.** The kinetics of FMLP-induced CR3 up-regulation and aggregation of human neutrophils. Neutrophils isolated at 4°C were warmed to 37°C for 10 min and then further incubated in Hapes buffer with or without 10<sup>-7</sup> M FMLP

for the times indicated along the x axis. Cells were then subjected to immunofluorescence staining and cytofluorographic analysis. FMLP-attributable CR3 up-regulation (open circles), expressed in RFU, was defined as the relative fluorescence of cells exposed to FMLP minus that of control cells warmed in buffer alone. FMLP-induced aggregation was measured as described in Methods. Aggregation (closed circles) is expressed as the percent maximal transmission of light measured at discrete times after the addition of FMLP (x axis). Results are given as the mean±SEM for three experiments.



**Figure 4.** The effect of extracellular calcium and magnesium on FMLP-induced neutrophil CR3 expression and aggregation. Neutrophils were isolated from blood anticoagulated with 10 mM EDTA, washed, and suspended in buffer with or without 1.2 mM  $\text{CaCl}_2$  and 1.3 mM

$\text{MgCl}_2$ . After exposing these cells to  $10^{-7}$  M FMLP for 1 or 30 min, they were subjected to immunofluorescence staining and cytofluorographic analysis. Results (RFU) are expressed as mean  $\pm$  SEM for five experiments. The inset shows representative curves for  $10^{-7}$  M FMLP induced aggregation of the same cells in the presence or absence of extracellular divalent cations.

all stimuli examined, the ionophore ionomycin ( $2 \times 10^{-7}$  M) elicited both the greatest CR3 up-regulation and the most aggregation.  $\text{LTB}_4$  ( $2 \times 10^{-7}$  M) and ZAS (10% vol/vol), agents that engage specific receptors, had intermediate effects similar to those of FMLP.

**Pharmacologic agents.** We next evaluated the effects of pharmacologic agents known to inhibit discrete neutrophil functions (31, 32) on CR3 up-regulation and neutrophil aggregation. Fig. 5 shows the effects of pretreating neutrophils with sodium salicylate or tetracaine on these two responses. 3 mM sodium salicylate had no effect on the up-regulation of CR3 attributable to a 1-min exposure to  $10^{-7}$  M FMLP ( $45 \pm 4$  vs.  $40 \pm 7$  RFU, NS). After 30 min of FMLP exposure, CR3 up-regulation was significantly enhanced in the presence of sodium salicylate ( $258 \pm 20$  vs.  $214 \pm 20$  RFU,  $P < 0.005$ ). In contrast to CR3 up-regulation, aggregation was significantly inhibited ( $55 \pm 4\%$  control,  $P < 0.01$ ) in the presence of 3 mM sodium salicylate (Fig. 5). At concentrations that resulted in 50% inhibition of neutrophil aggregation, piroxicam and indomethacin had no effect on up-regulation of CR3 (data not shown). Preincubation of neutrophils in buffer containing 1

mM tetracaine for 10 min at  $37^\circ\text{C}$  had converse effects (Fig. 5). CR3 alpha chain up-regulation was abolished after 1 min and  $68 \pm 5\%$  inhibited ( $P < 0.0001$ ) after 30 min of FMLP exposure. Similarly, using CLB-LFA-1-1, no up-regulation of CR3 beta chain could be detected after a 1-min exposure to FMLP ( $116 \pm 4$  vs.  $115 \pm 13$  RFU). In contrast to the absence of up-regulation of either the alpha or beta chain of CR3, aggregation was significantly enhanced ( $122 \pm 8\%$  control,  $P < 0.05$ ) in neutrophils pretreated with 1 mM tetracaine.

**Neutroplasts.** Neutroplasts are in vitro constructs consisting of neutrophil plasma membrane enclosing cytoplasm devoid of granules. Because they remain metabolically active, they serve as a useful model for neutrophil function in the absence of degranulation (33). Fig. 6 A shows that neutroplasts specifically expressed CR3 on their surface. However, unlike intact neutrophils, neutroplasts did not increase surface expression of CR3 after exposure to  $10^{-7}$  M FMLP for 1 min ( $104 \pm 7$  vs.  $106 \pm 3$  RFU, NS) or 30 min ( $104 \pm 13$  vs.  $109 \pm 13$  RFU, NS). Exposure of neutroplasts to FMLP for 1 min had similar effects on CR3 beta chain expression ( $112 \pm 5\%$  control). Fig. 6 B shows that, despite their inability to up-regulate CR3, neutroplasts were capable of aggregation upon exposure to FMLP. Moreover, this aggregation was CR3 dependent: pretreatment of neutroplasts with the anti-CR3 MABs, MN-41 or CLB-LFA-1-1, effectively inhibited FMLP-induced aggregation.

## Discussion

Several lines of evidence have established that the neutrophil iC3b receptor, CR3, is required for normal cell adhesion (9, 11, 12, 16–18). Nevertheless, the mechanism by which the CR3 molecule promotes cellular adhesion remains to be elucidated. That all degranulating stimuli that enhance neutrophil adhesion also induce significant up-regulation of surface CR3 has led to the hypothesis that adhesiveness depends upon the quantity of CR3 expressed on the cell surface (8, 9, 12, 22, 23). This concept is supported by the observation that the clinical severity of CR3/LFA-1/gp150/95 deficiency is directly pro-

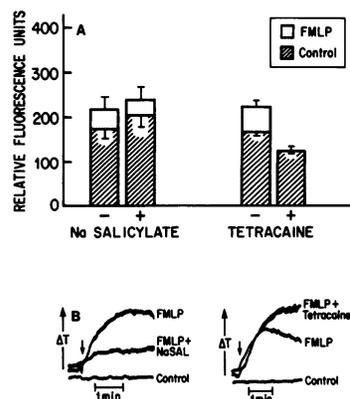
**Table II.** Relative Responses (Aggregation and CR3 Up-regulation) of Neutrophils Exposed to Various Agents for 1 Min

Agent	Relative responses*	
	Aggregation <sup>†</sup> (n = 5)	CR3 up-regulation <sup>‡</sup> (n = 3)
Ionomycin (200 nM)	100	100
FMLP (100 nM)	$58 \pm 4$	$48 \pm 10$
$\text{LTB}_4$ (200 nM)	$48 \pm 6$	$44 \pm 10$
ZAS (10% vol/vol)	$42 \pm 6$	$52 \pm 5$
A23187 (10 $\mu\text{M}$ )	$16 \pm 1$	$79 \pm 12$

\* Percent response relative to that obtained with ionomycin, which gave maximal responses.

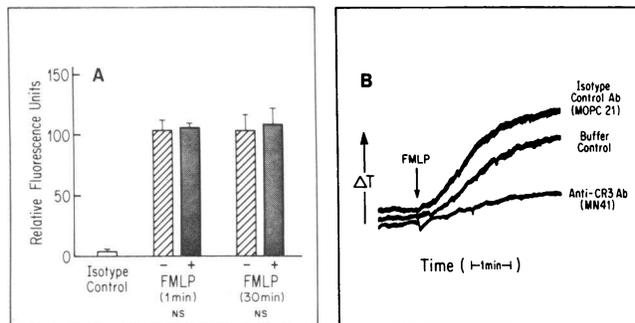
<sup>†</sup> Measured as described in Methods and quantitated as the area (in squared centimeters) under each aggregation curve in the first minute after exposure to the agent.

<sup>‡</sup> Relative fluorescence was measured as described in Methods. Up-regulation was defined as the RFU of cells exposed to each agent for 1 min minus that of control cells warmed in buffer alone.



**Figure 5.** The effects of sodium salicylate and tetracaine on FMLP-induced neutrophil CR3 expression (A) and aggregation (B). Neutrophils were incubated in HEPES buffer with or without 3 mM sodium salicylate or 1 mM tetracaine. (A) Neutrophils were exposed to  $10^{-7}$  M FMLP or buffer alone for 1 min and then subjected to indirect immunofluorescence staining and cytofluorographic analysis. Results (RFU) are

expressed as mean  $\pm$  SEM of four experiments. (B) Representative  $10^{-7}$  M FMLP-induced aggregation curves for the same neutrophils in the presence or absence of 3 mM sodium salicylate or 1 mM tetracaine.



**Figure 6.** Neutrophil CR3 expression (A) and aggregation (B). Neutrophils were prepared as described in Methods. (A) CR3 expression on neutrophils exposed to  $10^{-7}$  M FMLP or buffer alone for 1 or 30 min was measured by indirect immunofluorescence staining and cytofluorographic analysis. Results (RFU) are given as the mean  $\pm$  SEM ( $n = 4$ ). Specific surface staining of CR3 was confirmed by the negative results for neutrophils stained with an isotype control MAb (MOPC-21). (B) Representative ( $n = 3$ )  $10^{-7}$  M FMLP-induced aggregation curves of neutrophils preincubated with saturating concentrations of anti-CR3 MAb (MN-41), isotype control MAb (MOPC-21), or buffer alone.

portional to the amount of CR3 expressed on the deficient neutrophils (14).

However, several recent observations suggest that the quantitative hypothesis may not be correct. O'Shea et al. (24) showed that neutrophils did not up-regulate CR3 upon exposure to FMLP, whereas Korchak et al. (33) showed that neutrophils were capable of FMLP-induced aggregation and superoxide anion generation. Lopez et al. (34) demonstrated that recombinant human granulocyte-macrophage colony-stimulating factor enhanced the surface expression of neutrophil CR3 but did not promote adherence of neutrophils to endothelial cells or plastic surfaces, functions that have been shown to be CR3 dependent (17, 35). Conversely, Vedder and Harlan (36) have reported that, although neutrophils treated with the anion channel blocker, 4,4'-diisocyanostilbene-2,2'-disulfonic acid, failed to up-regulate CR3 upon exposure to PMA or A23187, their increased adhesion to endothelial cells was undiminished. In studies designed to elucidate the adhesive mechanism of the related molecule, LFA-1, Rothlein and Springer (37) showed that PMA-induced aggregation of an EBV-transformed B lymphoblastoid cell line was LFA-1 dependent, but not associated with an increase in surface expression of LFA-1.

Our studies provide additional evidence that CR3-mediated neutrophil aggregation is not dependent on the quantity of new molecules translocated to the cell surface. Neutrophils isolated at  $4^{\circ}\text{C}$  and rewarmed to  $37^{\circ}\text{C}$  in the absence of chemoattractant increased CR3 expression twofold, but did not aggregate as assessed by aggregometry or microscopic analysis. The absence of aggregation under these conditions could not be explained by failure to attain a threshold quantity of surface CR3, because the quantity of CR3 expressed on neutrophils rewarmed for 50 min (no aggregation) was identical to that expressed on cells exposed to FMLP for 1 min (maximal aggregation).

Physiologically significant neutrophil responses such as degranulation, superoxide anion generation, and aggregation are initiated within seconds and complete within 2 min of

exposure of the cells to a variety of stimuli (38, 39). We have analyzed the kinetics of CR3 up-regulation as early as 15 s after stimulation. Our results, which agree with those of Miller et al. (22), whose earliest timepoint was 2 min, show marked discordance between the kinetics of FMLP-induced CR3 up-regulation and those of neutrophil aggregation. Not only was relatively little ( $26 \pm 7\%$ ) of the CR3 translocatable by FMLP brought to the surface of the cell at times when aggregation was maximal (1 min), but new CR3 molecules continued to be translocated to the plasma membrane for 20 to 40 min after the addition of chemoattractant, long after aggregation had reversed. The latter observation excludes the quantity of surface CR3 from being the sole determinant of neutrophil adhesiveness.

A third line of evidence that dissociates neutrophil aggregation from CR3 up-regulation is the discordant extracellular calcium and magnesium requirements for these two responses. As others have reported (40), we found that extracellular divalent cations are required for maximal up-regulation of CR3. Nevertheless, in the absence of extracellular calcium and magnesium, we found a twofold increase in expression of CR3 on FMLP-treated cells compared with controls. In comparison, neutrophil aggregation was absolutely dependent on extracellular divalent cations. The latter observation is concordant with studies demonstrating a requirement for magnesium in LFA-1-dependent functions (e.g., adhesion of cytotoxic T cells [41] and aggregation of EBV-transformed B lymphoblastoid cells [37]) and the binding of iC3b to CR3 (42). Whereas one explanation for our observation may be that the molecular interaction between CR3 and its adhesive ligand requires the presence of divalent cations, an alternative explanation is that extracellular calcium and/or magnesium are required for the activation of the CR3 molecule, but not its translocation to the plasma membrane.

The different relative potencies of various stimuli in eliciting CR3 up-regulation and aggregation represent a fourth line of evidence that dissociates these two responses. Since A23187 was twice as potent as FMLP in eliciting CR3 up-regulation, yet less than one-third as potent in promoting aggregation, neutrophil aggregation is unlikely to result simply from the up-regulation of CR3.

Our experiments with sodium salicylate, tetracaine, and neutrophils further dissociate neutrophil up-regulation of CR3 and aggregation. Sodium salicylate and other nonsteroidal anti-inflammatory drugs have been shown to inhibit neutrophil aggregation (31) by a mechanism independent of their effect on cyclooxygenase (43). Our current studies demonstrate that these agents do not inhibit up-regulation of CR3. Up-regulation of CR3 thus is not sufficient to elicit maximal aggregation. This conclusion is supported by our observation, reported elsewhere (44), that resting neutrophils treated with MN-41 and then thoroughly washed were incapable of aggregation, despite normal CR3 up-regulation in response to FMLP.

Studies with tetracaine and neutrophils indicate that the converse is also true. Local anesthetics have various effects on neutrophil function (32, 45). We have shown that pretreatment with tetracaine did not inhibit neutrophil aggregation. In striking contrast, tetracaine completely prevented up-regulation of CR3 in response to a 1-min exposure to FMLP. As has been reported (24), neutrophils did not up-regulate CR3. However, not only were neutrophils capable of aggregation in

response to chemoattractant, but this aggregation was inhibited by prior exposure to anti-CR3 MAbs. These two independent observations demonstrate that up-regulation of CR3 is not required for aggregation.

Together, these studies support the conclusion that, although constitutive CR3 on the plasmalemma is required for neutrophil aggregation, up-regulation of CR3 is neither necessary nor sufficient to promote the cell-cell adhesion induced by chemoattractant. CR3 or the structure to which it binds probably requires activation to promote adhesion. This step might involve a biochemical modification or events such as a positional change of the receptor within the plane of the membrane.

It is known from other studies that the capacity of CR3 to bind iC3b-coated particles and to promote phagocytosis is not constitutive but requires activation. Wright and Silverstein showed that binding of C3-coated particles to monocyte CR1 or CR3 did not promote phagocytosis of the particles, unless the complement receptors were in an activated state induced by PMA, which did not correlate with receptor number (42). Subsequent studies revealed that treatment of cultured monocytes with recombinant IFN gamma reduced iC3b binding activity without affecting the number of CR3 molecules expressed on the cell surface (46). Griffin and Mullinax showed that the ability of murine macrophage C3b receptors to promote phagocytosis was associated with their ability to move laterally within the plane of the membrane (47). Similar results were recently reported in neutrophil studies using colloidal gold-labeled anti-CR3 MAbs, which revealed that PMA-induced increased neutrophil binding of iC3b-coated particles was associated with clustering of CR3 molecules on the cell surface (48). Earlier studies with albumin-coated latex beads showed that FMLP-induced changes in neutrophil adhesiveness were associated with a movement of adhesive sites toward the uropod (49). Wright and Meyer showed that PMA induced a sequential rise and subsequent fall in neutrophil binding of iC3b-coated erythrocytes that did not correlate with CR3 expression. These authors conclude that CR3 activation, defined as the ability to promote phagocytosis of bound iC3b-coated particles, results from a qualitative rather than a quantitative change in CR3. Based on indirect evidence using thiophosphate-loaded neutrophils, these authors suggest that this molecular activation/deactivation may involve phosphorylation and subsequent dephosphorylation (50). However, direct evidence of neutrophil CR3 phosphorylation is lacking (51). Our studies indicate that CR3-dependent homotypic aggregation, like CR3-dependent phagocytosis, requires an activation event. Elucidation of the processes that regulate CR3-mediated functions will require further study.

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