THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 1989 by The American Society for Biochemistry and Molecular Biology, Inc.

Vol. 264, No. 36, Issue of December 25, pp. 21711-21719, 1989
Printed in U.S.A.

Platelet-activating Factor Enhances Complement-dependent Phagocytosis of Diamide-treated Erythrocytes by Human Monocytes through Activation of Protein Kinase C and Phosphorylation of Complement Receptor Type One (CR1)*

(Received for publication, November 16, 1988)

Federico Bussolino, Elisabeth Fischer‡, Franco Turrini, Michel D. Kazatchkine‡, and Paolo Arese§

From the Dipartimento di Genetica, Biologia e Chimica Medica, Università di Torino, Torino 10126, Italy and ‡Institut National de la Santé et de la Recherche Médicale U28, Hôpital Broussais, Paris 75674, France

Oligomerization of band 3 protein has been recently indicated as an early event in senescent or damaged red cell membrane followed by specific deposition of anti-band 3 antibodies and binding of complement C3 fragments. The band 3-anti-band 3-C3b complex is recognized by homologous monocytes, and phagocytosis ensues. This study shows that recognition of the anti-band 3-C3b complex by the monocyte C3b receptor type one (CR1) plays a crucial role in the process of removal of damaged red cells.

Indeed, blocking of monocyte CR1 with an anti-CR1 monoclonal antibody abrogated phagocytosis of diamide-treated red cells. Platelet-activating factor (PAF) is a phospholipid mediator involved in inflammatory processes. Nanomolar (*R*)-PAF enhanced the CR1-dependent phagocytosis of diamide-treated human red cell and of sheep red cells coated with C3b, induced the fast translocation of protein kinase C to monocyte membrane compartment, and stimulated the phosphorylation of monocyte CR1. The biologically inert lyso-PAF and the enantiomer (*S*)-PAF were inactive. PAF receptor antagonists and inhibitors of protein kinase C blocked the enhancement of phagocytosis induced by PAF. Protein kinase C translocation, phosphorylation of CR1, and stimulation of this receptor to an active state capable of mediating phagocytosis represent a novel pathway by which PAF interferes with red cell homeostasis and possibly modulates inflammatory reactions and host mechanisms against infections.

Phagocytosis of opsonin-coated cellular targets is mediated by receptors for the immunoglobulin Fc domain and for the complement cleavage products C3b, iC3b, and possibly C3dg/C3d (1–3). Fc receptors readily initiate ingestion of IgG-coated targets, whereas C3 receptors on resting cells mediate target attachment. CR1 and CR3 complement receptors on monocytes acquire phagocytic capability following stimulation of the cells with T cell lymphokines (4), phorbol esters (5), and extracellular matrix proteins (6–8).

Mononuclear phagocytes eliminate senescent or damaged red blood cells (RBC)[1] while leaving mature viable cells unharmed. The specific membrane marker that provides the signal for recognition and removal of senescent or damaged RBC by phagocytic cells has remained controversial (9, 10). A recently proposed mechanism (11–13) includes oligomerization of band 3 protein as the primary event occurring on the senescent or damaged RBC membrane. The latter step is followed by specific deposition of naturally occurring anti-band 3 antibodies and secondary binding of C3 fragments through activation of the alternative complement pathway. The band 3- anti-band 3-C3 complex is recognized by homologous monocytes, and phagocytosis ensues (11–13).

The biochemical steps accounting for the transition of CR1 and CR3 from the resting to the activated state are unknown. However, the ability of phorbol esters to induce activation of CR1 and to promote phosphorylation of the receptor (14) suggests a role for protein kinase C (PKC) in inducing C3-mediated phagocytosis.

Platelet-activating factor (1-*O*-alkyl-(*R*)-acetyl-glycero-3-phosphocholine, PAF) is an autocrine lipid mediator involved in the inflammatory reaction. PAF is produced by and acts on platelets, neutrophils, monocytes/macrophages, and endothelial cells (15, 16). PAF stimulates the production of reactive oxygen intermediates in macrophages (17) concomitant with the rise of intracellular Ca^{2+} (18) and the activation of inositol lipid hydrolysis (19). Macrophages produce and release PAF during phagocytosis of aggregated IgG or of C3-coated bakers' yeast (20, 21). Although evidently involved in inflammation, no data point to PAF as a modulator of the phagocytic response.

In this study, we show that PAF specifically enhances the capability of the monocyte to ingest human RBC (RBC^{hu}) treated with low doses of diamide and opsonized in whole serum, a model of complement-dependent removal of RBC damaged by oxidant injury (11–13). PAF-mediated enhancement of ingestion is suppressed by preincubation of monocytes with anti-CR1 antibodies. PAF does not affect Fc-

* This work was supported by Consiglio Nazionale delle Ricerche, Roma, Italy Grant 87.00834.51 (Special Project on the Molecular Basis of Inherited Disease) (to P. A.); by grants (to M. K.) from the Institut National de la Santé et de la Recherche Médicale (INSERM), Paris, France; and by a short term fellowship (to E. F.) from the Institute for Scientific Interchange, Torino, Italy. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be sent: Dipartimento di Genetica, Biologia e Chimica Medica, Università di Torino, Via Santena 5 bis, Torino 10126, Italy.

[1] The abbreviations used are: RBC, red blood cells; RBC^{hu}, human RBC; RBC^{s}, sheep RBC; $RBC^{s}C3b$, RBC^{s} bearing C3b; ANOVA, analysis of variance; BSA, bovine serum albumin; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; mAb, monoclonal antibody; PBS, phosphate-buffered saline; PKC, protein kinase C; SDS, sodium dodecyl sulfate; PAF, platelet-activating factor; Me_2SO, dimethyl sulfoxide.

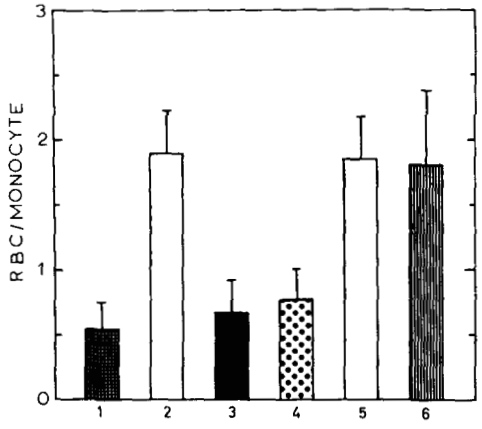

FIG. 1. **Phagocytosis of RBC^{hu} by adherent monocytes.** Adherent monocytes (7×10^4) were incubated for 30 min at 37 °C in 2 ml of RPMI 1640-BSA with 20 μg/ml anti-CR1 mAb J3D3 or with anti-CR3 mAb Mo1 or with mouse nonimmune IgG or with medium, then washed twice with 2 ml of RPMI 1640 containing 1% BSA. Phagocytosis was initiated by adding 16×10^6 RBC suspended in 1 ml of RPMI 1640 and stopped after 1 h of incubation at 37 °C in a 5% CO_2 humidified incubator. Phagocytosis was by monocytes of RBC^{hu} incubated in whole serum (*lane 1*); RBC^{hu} treated with 20 μM diamide and incubated in whole serum (*lane 2*); RBC^{hu} treated with 20 μM diamide and incubated in diisopropyl fluorophosphate-treated serum (*lane 3*). Phagocytosis of RBC^{hu} treated with diamide and incubated in whole serum was by adherent monocytes preincubated with mAb J3D3 (*lane 4*); mAb Mo1 (*lane 5*); and mouse nonimmune IgG (*lane 6*). Results are the mean values ± S.D. of 10 experiments done in triplicate. *p*, calculated by two-way ANOVA, was 0.00001. The Student-Newman-Keuls test gave $p < 0.05$ for the following comparisons (numbers refer to *lanes*): 6 *versus* 1, 6 *versus* 3, 6 *versus* 4, 5 *versus* 1, 5 *versus* 3, 5 *versus* 4, 2 *versus* 1, 2 *versus* 3, 2 *versus* 4.

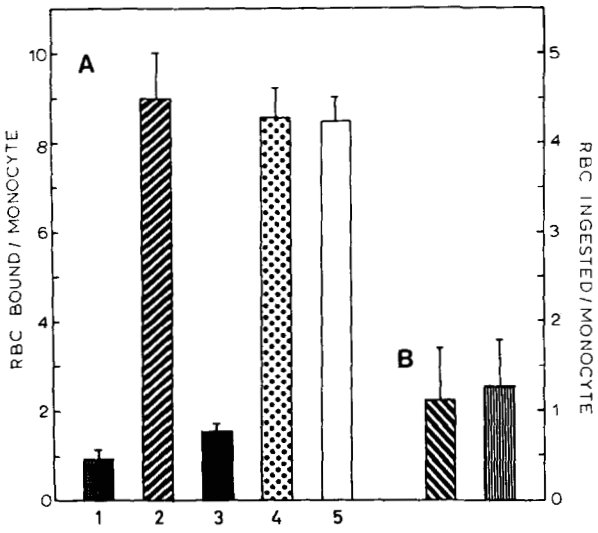

FIG. 2. **Attachment and phagocytosis of RBC^{s}C3b by adherent monocytes.** Adherent monocytes (7×10^4) were treated as detailed in the legend to Fig. 1. *A*, attachment to monocytes of RBC^s resuspended in PBS (*lane 1*); RBC^{s}C3b resuspended in PBS (*lane 2*). Attachment of RBC^{s}C3b to monocytes pretreated with mAb J3D3 (*lane 3*); mAb Mo1 (*lane 4*); and nonimmune IgG (*lane 5*). Results are the mean values ± S.E. of five experiments done in duplicate. *p*, calculated by two-way ANOVA, was 0.00001. The Student-Newman-Keuls test gave $p < 0.05$ for the following comparisons (numbers refer to *lanes*): 2 *versus* 1, 2 *versus* 3, 4 *versus* 1, 4 *versus* 3, 5 *versus* 1, 5 *versus* 3. *B*, phagocytosis by monocytes of RBC^{s}C3b resuspended in PBS (▨); RBC^s resuspended in PBS (▩). Results are the mean values ± S.D. of five experiments done in duplicate. *p*, calculated by two-way ANOVA, was 0.1408.

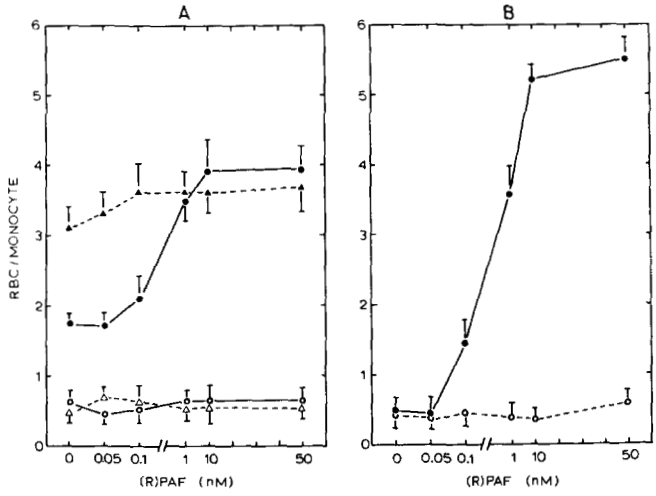

FIG. 3. **Phagocytosis of erythrocytes by adherent monocytes treated with (*R*)-PAF.** Adherent monocytes (7×10^4) were incubated at 37 °C with different concentrations of (*R*)-PAF. After 10 min, the medium was removed, the monocytes washed once with 2 ml of PBS and refed with RPMI 1640. Phagocytosis was initiated by adding 16×10^6 RBC suspended in 1 ml of RPMI 1640 and stopped after 1 h of incubation at 37 °C in a 5% CO_2 humidified incubator (see "Experimental Procedures"). *Panel A*; phagocytosis of RBC^{hu}. O——O, RBC^{hu} incubated in whole serum. *p*, calculated by two-way ANOVA, was 0.2328. ●——●, RBC^{hu} treated with 20 μM diamide and incubated in whole serum. $p = 0.00001$. The Student-Newman-Keuls test gave $p < 0.05$ for the following comparisons: monocytes treated with 50 nM (*R*)-PAF *versus* monocytes treated with 0, 0.05, and 0.1 nM (*R*)-PAF; monocytes treated with 10 nM (*R*)-PAF *versus* monocytes treated with 0, 0.05, and 0.1 nM (*R*)-PAF; monocytes treated with 1 nM (*R*)-PAF *versus* monocytes treated with 0, 0.05, and 0.1 nM (*R*)-PAF; △- - -△, RBC^{hu} treated with 20 μM diamide and incubated in diisopropyl fluorophosphate-treated serum. $p = 0.0836$. ▲- - -▲, RBC^{hu} opsonized with anti-D IgG. $p = 0.0535$. Results are the mean values ± S.D. of four experiments done in triplicate. *Panel B*, phagocytosis of RBC^s. O- - -O, RBC^s incubated in PBS. $p = 0.2099$. ●——●, RBC^{s}C3b incubated in PBS. $p = 0.00001$. The Student-Newman-Keuls test gave $p < 0.05$ for the following comparisons: monocytes treated with 50 nM (*R*)-PAF *versus* monocytes treated with 0, 0.05, 0.1, 1, and 10 nM (*R*)-PAF; monocytes treated with 10 nM (*R*)-PAF *versus* monocytes treated with 0, 0.05, 0.1, and 1 nM (*R*)-PAF; monocytes treated with 1 nM (*R*)-PAF *versus* monocytes treated with 0, 0.05, and 0.1 nM (*R*)-PAF; monocytes treated with 0.1 nM (*R*)-PAF *versus* monocytes treated with 0 and 0.05 nM (*R*)-PAF. Results are the mean values ± S.D. of four experiments done in triplicate.

TABLE I

Adherence of RBC^{hu} and RBC^s to (R)-PAF-treated human monocytes

Adherent monocytes (7×10^4) were incubated for 10 min at 37 °C with or without 10 nM (*R*)-PAF. RBC adhesion was assayed as described under "Experimental Procedures." Results are the mean values ± S.D. of three experiments done in duplicate.

	(*R*)-PAF	Control	*p*[a]
	RBC bound/monocyte		
Diamide-treated RBC^{hu} incubated in PBS[b]	0.7 ± 0.1	0.8 ± 0.4	0.4356
Diamide-treated rBC^{hu} incubated in serum	3.8 ± 0.4	3.1 ± 0.7	0.2135
RBC^s incubated in PBS	1.0 ± 0.2	0.9 ± 0.5	0.0891
RBC^{s}C3b incubated in PBS	9.4 ± 1.6	9.0 ± 1.1	0.0717

[a] *p* was calculated by two-way ANOVA.

[b] RBC were incubated in PBS or serum as described under "Experimental Procedures."

TABLE II

Effect of (R)-PAF and PAF-like molecules on phagocytosis by adherent monocytes

Adherent monocytes (7×10^4) were incubated for 10 min at 37 °C with or without additions before initiation of phagocytosis. Results are the mean values ± S.D. of three experiments done in triplicate.

Additions	Diamide-treated RBC^{hu}[a]	RBC^{hu}	$RBC^{s}C3b$	RBC^{s}
		ingested RBC/monocyte		
1. None	1.7 ± 0.9	0.6 ± 0.2	0.7 ± 0.4	0.6 ± 0.2
2. (*R*)-PAF (10 nM)	3.8 ± 0.8	0.8 ± 0.3	5.9 ± 0.3	0.7 ± 0.2
3. (*S*)-PAF (100 nM)	1.6 ± 0.6	0.8 ± 0.9	0.3 ± 0.2	0.8 ± 0.2
4. Lyso-PAF (1 μM)	1.9 ± 0.6	0.8 ± 0.2	0.8 ± 0.3	0.5 ± 0.3
	$p = 0.0028$[b]	$p = 0.4726$	$p = 0.0005$	$p = 0.6441$

[a] Diamide-treated and untreated RBC^{hu} were incubated in whole serum as described under "Experimental Procedures."

[b] For each column of data, p was calculated by two-way ANOVA. Whenever the ANOVA gave $p < 0.05$, the Student-Newman-Keuls test was applied for more specific comparisons. The following comparisons gave $p < 0.05$. Phagocytosis of diamide-treated RBC^{hu}: 2 *versus* 3, 2 *versus* 1, 2 *versus* 4, 4 *versus* 3; phagocytosis of $RBC^{s}C3b$: 2 *versus* 3, 2 *versus* 1, 2 *versus* 4, 4 *versus* 3.

TABLE III

Inhibition by PAF receptor antagonists of (R)-PAF-induced increase of phagocytosis

Adherent monocytes (7×10^4) were incubated in 1 ml RPMI-BSA for 10 min at 37 °C with or without PAF receptor antagonists before the addition of 10 nM (*R*)-PAF. Results are the mean values ± S.D. of three experiments done in duplicate.

Additions	% Inhibition of phagocytosis[a]	
	Diamide-treated RBC^{hu}	$RBC^{s}C3b$
BN52021[b]		
1. 0.1 μM	0	0
2. 1 μM	12.3 ± 1.7	6.0 ± 1.9
3. 10 μM	48.0 ± 8.9	52.8 ± 10.4
4. 50 μM	74.5 ± 6.9	78.1 ± 5.8
	$p = 0.0043$[c]	$p = 0.0057$
CV3988[d]		
1. 0.1 μM	0	0
2. 1 μM	3.6 ± 1.5	0
3. 10 μM	31.3 ± 4.1	25.6 ± 0.8
4. 50 μM	66.7 ± 6.4	79.1 ± 4.4
	$p = 0.0001$	$p = 0.0010$

[a] % Inhibition was calculated from the values of phagocytosis of diamide-treated RBC^{hu} incubated in whole serum (3.5, 2.7, 4.1 RBC/monocyte) and $RBC^{s}C3b$ (5.5, 6.3, 4.8 RBC/monocyte) by adherent monocytes stimulated with 10 nM (*R*)-PAF for 10 min at 37 °C.

[b] The phagocytosis of diamide-treated RBC^{hu} incubated in whole serum or of $RBC^{s}C3b$ by monocytes preincubated with 50 μM BN52021 was 0.7 ± 0.6 and 1.8 ± 0.6 RBC/monocyte, respectively. Me_2SO used to dissolve BN52021 did not interfere with phagocytosis (3.6 ± 0.8 diamide-treated RBC^{hu} and 5.1 ± 0.9 $RBC^{s}C3b$ were ingested, respectively, by monocytes treated with 0.01% Me_2SO).

[c] For each column of data, p was calculated by two-way ANOVA. Whenever the ANOVA gave $p < 0.05$, the Student-Newman-Keuls test was applied for more specific comparisons. The following comparisons gave $p < 0.05$. Phagocytosis of diamide-treated RBC^{hu} by monocytes preincubated with BN52021: 4 *versus* 1, 4 *versus* 2, 4 *versus* 3, 3 *versus* 1, 3 *versus* 2, 2 *versus* 1; phagocytosis of diamide-treated RBC^{hu} by monocytes preincubated with CV3988: 4 *versus* 1, 4 *versus* 2, 4 *versus* 3, 3 *versus* 1, 3 *versus* 2; phagocytosis of $RBC^{s}C3b$ by monocytes preincubated with BN52021: 4 *versus* 1, 4 *versus* 2, 4 *versus* 3, 3 *versus* 1, 3 *versus* 2; phagocytosis of $RBC^{s}C3b$ by monocytes preincubated with CV3988: 4 *versus* 2, 4 *versus* 1, 4 *versus* 3, 3 *versus* 2, 3 *versus* 1.

[d] The phagocytosis of diamide-treated RBC^{hu} incubated in whole serum or of $RBC^{s}C3b$ by monocytes preincubated with 50 μM CV3988 was 1.1 ± 0.3 and 1.7 ± 0.5, respectively.

mediated phagocytosis. In addition, PAF renders monocytic CR1 capable of mediating ingestion of sheep RBC (RBC^{s}) coated with human C3b ($RBC^{s}C3b$). PAF-induced phagocytosis of C3b-coated RBC correlates with translocation of PKC from the cytosol to the membrane and phosphorylation of CR1.

EXPERIMENTAL PROCEDURES

Reagents—1-*O*-Octadecyl-2-(*R*)-acetyl-glycero-3-phosphocholine ((*R*)-PAF) and 1-*O*-octadecyl-2-lyso-glycero-3-phosphocholine (lyso-PAF) were from Bachem Feinchemikalien (Bubendorf, Switzerland); [^{14}C]potassium cyanate (55 mCi/mmol), [^{32}P]orthophosphoric acid (40 mCi/ml), and [^{32}P]ATP (2 mCi/mmol) were from Amersham International; protein A-Sepharose was from Pharmacia LKB Biotechnology Inc.; XAR X-Omat films were from Kodak; CV3988 was from Takeda Chemical Industries (Osaka, Japan); H7 was from Seikagaku Co. (Miami, FL); staurosporin was from Kamiya Biomedical Co. (Thousand Oaks, CA); BN52021 was from Institut Henri Beaufour (Le Plessis Robinson, France); culture media were from Flow Laboratories (McLean, VA); plastics were from Falcon Labware (Oxnard, CA). 1-*O*-Octadecyl-2-(*S*)-acetyl-glycero-3-phosphocholine (enantiomer *S* form) was a gift of Drs. H. P. Kertscher and G. Ostermann (Karl Marx University, Leipzig and Medical Academy, Erfurt, German Democratic Republic), and human anti-D-specific IgG was a gift of Dr. H. U. Lutz (ETH Zentrum, Zurich, Switzerland). The other reagents were from Sigma or Merck. BN52021 and staurosporin were dissolved in dimethyl sulfoxide.

Mouse monoclonal (mAb) anti-CR1 antibody J3D3 and rabbit anti-CR1 antibodies were obtained by immunizing mice and rabbit with human CR1 (CD35) and were purified as described (22–24). Rabbit IgG was prepared from anti-CR1 antiserum and from normal rabbit serum with ammonium sulfate precipitation and DEAE-52-cellulose purification. Purified mouse and rabbit anti-CR1 IgG showed one band upon SDS-polyacrylamide gel electrophoresis analysis. Both antibodies immunoprecipitated two CR1 allotypic bands from RBC^{hu} membrane as described previously (22). Rabbit antibodies inhibited C3b-dependent rosette formation. mAb J3D3 is a reference antibody of the CD35 cluster for fourth human leukocyte differentiation antigens workshop. Anti-CR3 mAb (Mo1) was purchased from Coulter (Milano, Italy).

Buffers and Incubation Media—Buffers were as follows: phosphate-buffered saline (PBS; 150 mM NaCl, 10 mM Na_2HPO_4/NaH_2PO_4, pH 7.4), buffer A (10 mM HEPES, 10 mM β-mercaptoethanol, 5 mM EDTA, pH 7.5), buffer B (buffer A containing 0.24 M sucrose, 0.43 mM phenylmethylsulfonyl fluoride, 0.1 mM leupeptin, 0.005 mM pepstatin and 22 units/ml aprotinin), RIPA buffer (150 mM NaCl, 10 mM Na_2HPO_4/NaH_2PO_4, 0.68 mM sucrose, 5 mM EDTA, 10 mM NaF, 5 mM sodium pyrophosphate, 1 mM sodium vanadate, 1 mM ATP, 5 mM phenylmethylsulfonyl fluoride, 0.002 mM leupeptin, 0.002 mM pepstatin, 100 μg/ml DNase, 5% fetal calf serum, 2% Nonidet P-40, 0.2% SDS, pH 7.2), Tris buffer (40 mM Tris-HCl, 5 mM KCl, 116 mM NaCl, 0.2 mM $MgCl_2$, 5 mM D-glucose, pH 7.6), RPMI-fetal calf serum (RPMI 1640 supplemented with 10% fetal calf serum, pH 7.4), RPMI-BSA (RPMI 1640 supplemented with 0.25% BSA, pH 7.4), and RPMI-HEPES (RPMI 1640 containing 10 mM HEPES, pH 7.4).

Sheep Erythrocytes Bearing C3b—$RBC^{s}C3b$ were prepared by sequential deposition of C3b by fluid phase and cell-bound amplification convertase using purified human alternative complement pathway proteins C3, B and D as described previously (25). $RBC^{s}C3b$ were

TABLE IV

Effect of anti-CR1 and anti-CR3 antibodies on phagocytosis by nonstimulated and (R)-PAF-stimulated monocytes

Adherent monocytes (7×10^4) were preincubated for 30 min at 37 °C in 1 ml of RPMI-BSA with 20 μg/ml of different mAb or nonimmune IgG, washed twice with RPMI 1640 containing 1% BSA, then incubated 10 min with 10 nM (*R*)-PAF before initiation of phagocytosis. Phagocytosis was performed as described under "Experimental Procedures." Results are the mean ± S.D. of four experiments done in triplicate.

	Diamide-treated RBC^{hu} [a]	RBC^{hu}	$RBC^{s}C3b$	RBC^{s}
	ingested RBC/monocyte			
Nonstimulated monocytes				
1. None	1.9 ± 0.5	0.5 ± 0.2	0.7 ± 0.3	0.4 ± 0.3
2. Anti-CR1 (J3D3)	0.7 ± 0.3	0.6 ± 0.4	0.9 ± 0.5	0.3 ± 0.2
3. Anti-CR3 (Mol)	1.8 ± 0.6	0.3 ± 0.1	0.2 ± 0.4	0.4 ± 0.2
4. IgG	1.6 ± 0.8	0.7 ± 0.3	0.6 ± 0.8	0.5 ± 0.3
	$p = 0.0017$ [b]	$p = 0.7691$	$p = 0.5632$	$p = 0.7616$
(*R*)-PAF-stimulated monocytes				
1. None	3.8 ± 1.1	0.8 ± 0.3	5.8 ± 1.5	0.9 ± 0.4
2. Anti-CR1 (J3D3)	1.0 ± 0.5	0.6 ± 0.3	1.2 ± 0.6	0.7 ± 0.3
3. Anti-CR3 (Mol)	3.6 ± 0.5	0.3 ± 0.4	5.2 ± 2.1	0.8 ± 0.3
4. IgG	3.7 ± 0.4	0.5 ± 0.3	5.2 ± 1.7	0.9 ± 0.4
	$p = 0.0048$	$p = 0.2169$	$p = 0.0030$	$p = 0.2889$

[a] Diamide-treated and untreated RBC^{hu} were incubated in whole serum as described under "Experimental Procedures."

[b] For each column of data, *p* was calculated by two-way ANOVA. Whenever the ANOVA gave $p < 0.05$, the Student-Newman-Keuls test was applied for more specific comparisons. The following comparisons gave $p < 0.05$. Phagocytosis of diamide-treated RBC^{hu} by nonstimulated monocytes: 2 *versus* 1, 2 *versus* 3, 2 *versus* 4; phagocytosis of diamide-treated RBC^{hu} by (*R*)-PAF-stimulated monocytes: 1 *versus* 2, 4 *versus* 2, 3 *versus* 2; phagocytosis of $RBC^{s}C3b$ by (*R*)-PAF-stimulated monocytes: 1 *versus* 2, 4 *versus* 2, 3 *versus* 2.

coated with 40,000 molecules of C3b/cell as assessed by the binding of radiolabeled anti-C3 mAb (Bethesda Research Laboratories). For phagocytosis experiments, RBC^{s} and $RBC^{s}C3b$ were labeled with [^{14}C]cyanate (26).

Treatment of RBC^{hu}—RBC^{hu} were labeled with [^{14}C]cyanate as described previously (26) and resuspended at 25% hematocrit in Tris buffer and incubated with or without 20 μM diamide for 60 min at 37 °C. RBC were washed three times in Tris buffer and then incubated for 30 min at 37 °C and 33% hematocrit in autologous serum or diisopropyl fluorophosphate-treated autologuous serum (11). Alternatively, RBC^{hu} were opsonized at 33% hematocrit with 2 μg/ml human IgG anti-D dissolved in Tris buffer (11).

Preparation of Adherent Monocytes—Mononuclear cells were separated from human O Rh^+ blood collected in heparin by Lymphoprep gradient (27). 1×10^6 or 1×10^8 mononuclear cells were plated in 25- or 90-mm diameter Petri dishes, respectively (11). After a 60-min incubation, nonadherent cells were removed and incubated for 3 h in RPMI 1640 prior to starting the experiments. The contamination of lymphocytes estimated by nonspecific esterase staining (Sigma kit) never exceeded 4–5%. The number of adherent monocytes was estimated by DNA measurement (28).

PAF Treatment of Adherent Monocytes—Adherent monocytes were incubated with (*R*)-PAF, (*S*)-PAF, and lyso-PAF for the indicated periods of time and then washed three times with RPMI 1640. PAF receptor antagonists and PKC inhibitors were added 15 min prior to the addition of (*R*)-PAF phagocytosis. In some experiments, adherent monocytes were preincubated for 30 min at 37 °C in a 5% CO_2 humidified incubator with 10 μg/ml RPMI 1640 of mAb or control mouse IgG, washed three times with RPMI-HEPES containing 1% BSA, and incubated with RPMI 1640 (pH 7.4).

Phagocytosis Assay—2×10^7 [^{14}C]cyanate-labeled RBC^{hu} or $RBC^{s}C3b$ were plated on adherent monocytes. After a 60-min incubation at 37 °C, binding of different types of RBC to monocytes and phagocytosis were assessed as described (26).

Immunoprecipitation of CR1 from (R)-PAF-stimulated ^{32}P-Labeled Monocytes—6×10^7 adherent monocytes were depleted of intracellular phosphate by incubation for 1 h in phosphate-free Dulbecco's modified minimal essential medium and labeled with [^{32}P]phosphate (8 mCi at 1 mCi/ml) in the same medium for 1 h at 37 °C (14). After three washes, labeled cells were incubated with RPMI-HEPES alone or containing 10 nM (*R*)-PAF for 10 min at 37 °C. The cells were then lysed in RIPA buffer (14). Cell lysates were incubated on ice for 30 min, and insoluble material was removed by centrifugation at 25,000 × *g* for 20 min at 4 °C. Solubilized glycoproteins were sequentially precleared by incubation with protein A-Sepharose followed by incubation with normal rabbit IgG and removal of complexed proteins by protein A-Sepharose. After centrifugation (1,500 × *g*), precleared lysates were incubated with rabbit anti-CR1 IgG followed by the addition of protein A-Sepharose. The beads were washed with RIPA buffer, and adsorbed proteins eluted with 1% SDS for 5 min at 100 °C. The eluates were subjected to SDS-polyacrylamide gel electrophoresis on 7% polyacrylamide slab gels. The gels were dried and exposed to XAR X-Omat films with Cronex Xtra Intensifying Screens (Du Pont-New England Nuclear) at −70 °C for 5 days.

Partial Purification of PKC—1×10^7 adherent monocytes were washed three times with RPMI-BSA and then stimulated at 37 °C for different periods of time in 5 ml of prewarmed RPMI-BSA (pH 7.4) with the indicated concentrations of stimuli. In some experiments, adherent monocytes were preincubated for 10 min at 37 °C with 50 μM BN52021 or CV3988. At the end of the incubation, the cells were scraped in 5 ml of cold buffer B, sedimented, and resuspended in 1 ml of buffer B. After sonication (6 pulses of 10 s in ice bath, 100 watts, Labsonic 1510, B. Braun Melsungen, Federal Republic of Germany), cytosolic and particulate fractions were prepared by ultracentrifugation (29). The various cell fractions were applied to a DEAE-52-cellulose column (0.9 × 2.5 cm) equilibrated with buffer A (29). The kinase activity was eluted by application of an 18-ml linear concentration gradient of NaCl (0–0.2 M) in buffer A at flow rate of 0.2 ml/min.

PKC Assay—Total PKC activity was quantified by measuring the incorporation of ^{32}P from [^{32}P]ATP into type IIIS histone (30). The test mixture contained phosphatidylserine, diolein, $MgCl_2$, and $CaCl_2$ (30). Nonspecific PKC activity was assayed in the absence of cations and lipids but in the presence of EGTA (30). Net PKC activity was calculated as the difference between total and nonspecific PKC activities.

Statistical Analysis—For each experimental condition, 3–10 experiments were performed with different preparations of monocytes and RBC. Each measurement was repeated three times in order to assess the reproducibility of the obtained values. Some measurements were done with only two replicates, and the missing data were estimated from the mean within the same subgroup. The difference within the duplicate values never exceeded 3%. Two-way ANOVA with block of repeated measurements was applied to test the difference within groups and between blocks for each experiment. Furthermore, the analysis of variance for repeated measurements tested the significance of differences within sets of repeated measurements. By two-way, ANOVA, the differences within the repeated measurements were not significant in any experimental condition, and the means were used to summarize data for each block. Whenever the two-way ANOVA with repeated measurements was significant ($p < 0.05$), the block means were analyzed by the Student-Newman-Keuls test in order to

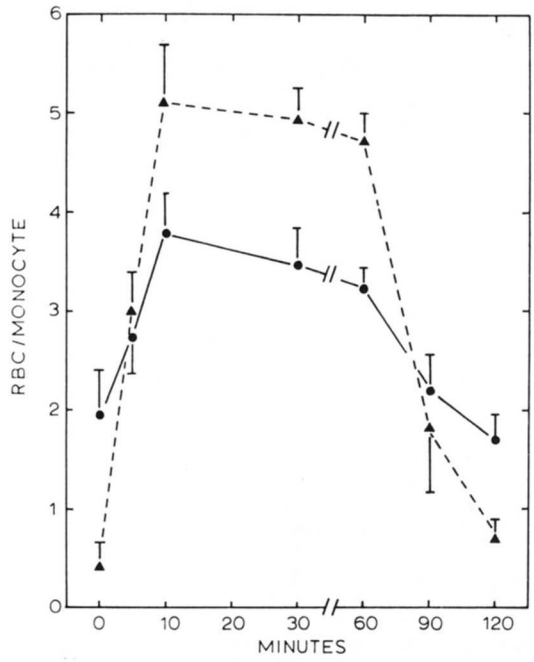

FIG. 4. **Time course of phagocytosis-enhancing effect of (*R*)-PAF on monocytes.** Adherent monocytes (7×10^4) were incubated at 37 °C for different periods of time with 10 nM (*R*)-PAF. The experiment was performed as detailed in the legends to Figs. 1 and 3. ●——●, RBC^{hu} treated with diamide and incubated in whole serum. *p*, calculated by two-way ANOVA, was 0.0001. The Student-Newman-Keuls test gave $p < 0.05$ for the following comparisons: monocytes stimulated for 10 min with (*R*)-PAF *versus* control and *versus* monocytes stimulated for 5 and 120 min; monocytes stimulated for 30 min *versus* control and *versus* monocytes stimulated for 120 min; monocytes stimulated for 60 min *versus* control. ▲- - -▲, $RBC^{s}C3b$ incubated in PBS. $p = 0.00001$. The Student-Newman-Keuls test gave $p < 0.05$ for the following comparisons: monocytes stimulated for 30 min with (*R*)-PAF *versus* control and *versus* monocytes stimulated for 120, 90, and 5 min; monocytes stimulated for 10 min *versus* control and *versus* monocytes stimulated for 120, 90, and 5 min; monocytes stimulated for 60 min *versus* control and *versus* monocytes stimulated for 120, 90, 5 min; monocytes stimulated for 5 min *versus* control and *versus* monocytes stimulated for 120 and 90 min; monocytes stimulated for 90 min *versus* control and *versus* monocytes stimulated for 120 min. Results are the mean values ± S.D. of three experiments done in triplicate.

make more specific comparisons among the different experimental conditions. These analyses were performed by using a BMDP2V software (BMDP Statistical Software, Inc., Los Angeles, CA).

RESULTS

Phagocytosis of RBC by Adherent Monocytes—RBC^{hu} treated with 20 μM diamide and incubated with normal human serum were phagocytosed by human adherent monocytes (Fig. 1). Heat (not shown) or diisopropyl fluorophosphate treatment of serum abrogated phagocytosis almost completely. Non-diamide-treated RBC^{hu} incubated with serum were not ingested. Preincubation of adherent monocytes with saturating amounts of anti-CR1 mAb J3D3 totally suppressed phagocytosis of diamide-treated RBC^{hu} incubated in whole serum. Phagocytosis was unaffected by pretreatment of monocytes with anti-CR3 mAb Mo1 or IgG from nonimmune mouse (Fig. 1).

$RBC^{s}C3b$ were attached to but not ingested by adherent monocytes. The ability of monocytes to bind $RBC^{s}C3b$ was inhibited by pretreatment of monocytes with anti-CR1 mAb Mo1 and was not affected by anti-CR3 mAb or nonimmune mouse IgG (Fig. 2).

Effect of (R)-PAF on the Phagocytosis of Diamide-treated RBC^{hu} and of $RBC^{s}C3b$—(*R*)-PAF enhanced phagocytosis of diamide-treated RBC^{hu} and $RBC^{s}C3b$ by monocytes in a dose-dependent manner (Fig. 3, *A* and *B*). The effect was already evident when adherent monocytes were pretreated with 1 nM (*R*)-PAF for 10 min and reached maximum values at 10–50 nM (*R*)-PAF. Adherence of diamide-treated RBC^{hu} and of $RBC^{s}C3b$ to monocytes was not affected by treating monocytes with 10 nM (*R*)-PAF (Table I). In addition, the amount of ^{125}I-labeled anti-CR1 mAb J3D3 that specifically bound to adherent monocytes in the presence of 50-fold excess antibody did not differ between untreated monocytes (3322 ± 950 cpm/well in triplicate experiments) and monocytes that had been treated with 10 nM (*R*)-PAF (3883 ± 2791 cpm/well) or 20 nM (*R*)-PAF (4287 ± 1015 cpm/well). (*R*)-PAF did not enhance the phagocytosis of RBC^{hu} opsonized with anti-D IgG (Fig. 3*A*). Enhancement of phagocytosis of diamide-treated RBC^{hu} incubated in whole serum and $RBC^{s}C3b$ by (*R*)-PAF was dependent on effect of (*R*)-PAF on monocytes since pretreatment of diamide-treated RBC^{hu} or $RBC^{s}C3b$ with (*R*)-PAF (1–100 nM, 10 min at 37 °C) had no effect on their ingestion (not shown).

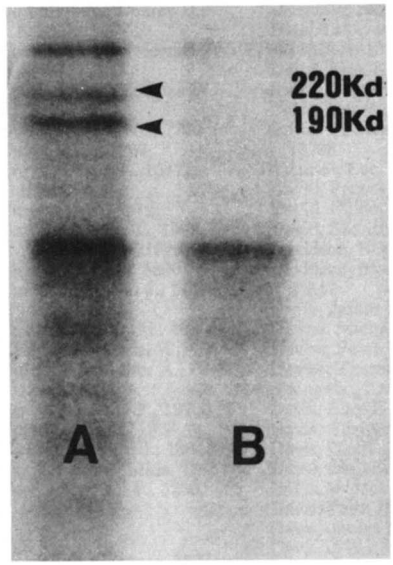

FIG. 5. **(*R*)-PAF-induced phosphorylation of CR1.** Autoradiography of SDS-polyacrylamide gel electrophoresis analysis of CR1 from ^{32}P-labeled adherent monocytes treated with 10 nM (*R*)-PAF (*A*) or with buffer alone (*B*) for 10 min at 37 °C.

No increase in ingestion of diamide-treated RBC^{hu} incubated in whole serum and of $RBC^{s}C3b$ was observed after treatment of monocytes with inactive PAF analogs such as lyso-PAF (1 μM) and (*S*)-PAF (100 nM) (Table II). Pretreatment of adherent monocytes with the specific PAF-receptor antagonists BN52021 and CV3988 inhibited the enhancement of phagocytosis in a dose-dependent manner (Table III). No enhancement of phagocytosis of diamide-treated RBC^{hu} incubated in whole serum and no phagocytosis of $RBC^{s}C3b$ was induced by (*R*)-PAF in monocytes that had been pretreated with anti-CR1 mAb J3D3 (Table IV). The time course of the effect of (*R*)-PAF on monocytes is shown in Fig. 4. Treatment of adherent monocytes with 10 nM (*R*)-PAF for 10 min induced a maximal enhancement of phagocytosis, whereas the effect declined progressively with an incubation period longer than 60 min. (*R*)-PAF (1–100 nM) had no effect on the number of monocytes that adhered to the culture dishes (not shown).

(R)-PAF-induced Phosphorylation of CR1—In order to examine whether the acquired ability of (*R*)-PAF-treated monocytes to ingest C3b-coated targets was associated with phos-

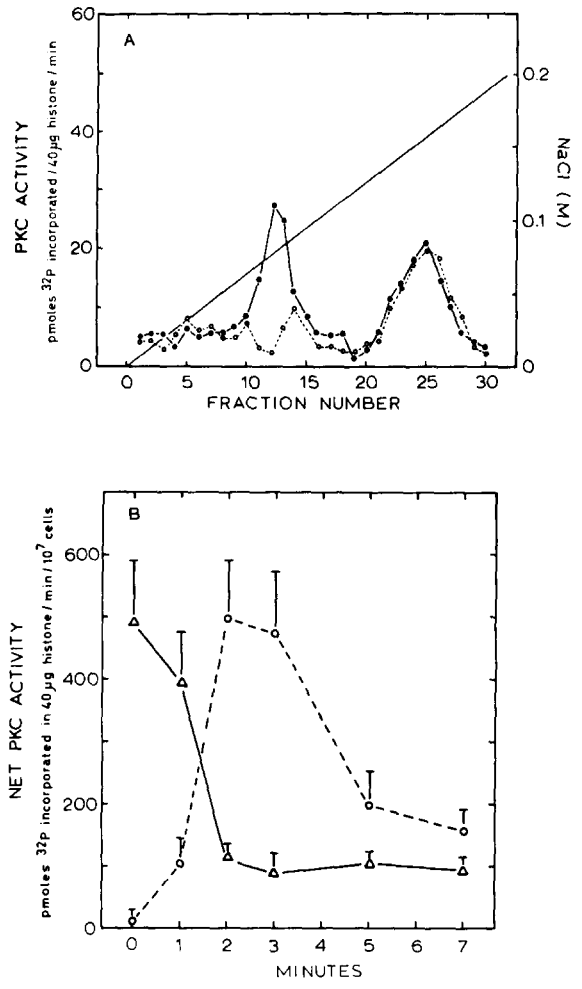

FIG. 6. **DEAE-52-cellulose chromatography profile of monocyte PKC and time course of PKC redistribution in adherent monocytes stimulated with (*R*)-PAF.** *Panel A*, DEAE-52-cellulose chromatography of PKC present in the soluble fraction. Adherent monocytes (2×10^7) were scraped with a rubber policeman in 5 ml of buffer B. After sonication (six pulses of 10 s in ice bath, 100 watts), particulate and soluble fractions were separated by centrifugation at 100,000 × *g* for 1 h at 4 °C. The supernatant and the pellet were resuspended in 1 ml of buffer B containing 1% Nonidet P-40 and applied to a DEAE-52-cellulose column (29) equilibrated with buffer A. After sample addition, the column was washed with 20 ml of buffer A, then eluted with a linear (0–0.2 M) NaCl gradient. The flow rate was 0.5 ml/min, and the fraction size was 2 ml. The conductivity of effluent solution was measured by an on-line conductivity meter. PKC activity was measured in 0.1-ml samples. The net PKC activity resulted from the difference of histone type III phosphorylation in the presence of Ca^{2+}, phosphatidylserine, and diolein, or of EGTA. *Panel B*, time course of (*R*)-PAF-induced PKC translocation. Adherent monocytes (2×10^7) were stimulated for different periods of time at 37 °C with 10 nM (*R*)-PAF in 5 ml of RPMI-BSA. The reaction was stopped by putting the dishes on ice. Each value was obtained by the addition of net PKC activity measured in the fractions of a DEAE-52 column eluted with 0.06–0.09 M NaCl. O– – –O, particulate fraction. *p*, calculated by two-way ANOVA, was 0.00001. The Student-Newman-Keuls test gave $p < 0.05$ for the following comparisons: control *versus* 2-, 3-, 5-, and 7-min stimulation; 1-min stimulation *versus* 2- and 3-min stimulation; 2-min stimulation *versus* 5- and 7-min stimulation; 3-min stimulation *versus* 5- and 7-min stimulation. Δ——Δ, soluble fraction. $p = 0.00001$. The Student-Newman-Keuls test gave $p < 0.05$ for the following comparisons: control *versus* monocytes stimulated for 2, 3, 5, and 7 min; 1-min stimulation *versus* 2-, 3-, 5-, and 7-min stimulation. Mean values ± S.D. of three experiments done in duplicate.

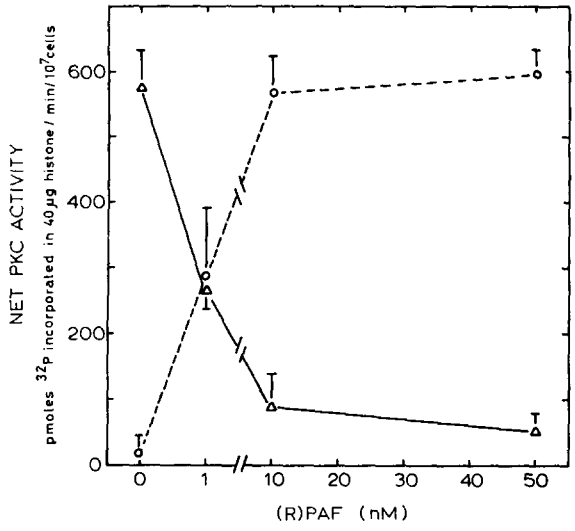

FIG. 7. **Dose-dependent effect of (*R*)-PAF-induced PKC activation.** Adherent monocytes (2×10^7) were stimulated for 3 min at 37 °C in 5 ml of RPMI-BSA with different concentrations of (*R*)-PAF and processed as described in the legend to Fig. 6. O– – –O, particulate fraction. *p*, calculated by two-way ANOVA, was 0.00001. The Student-Newman-Keuls test gave $p < 0.05$ for the following comparisons: monocytes stimulated with 10 nM (*R*)-PAF *versus* unstimulated and 1 nM (*R*)-PAF-stimulated monocytes; monocytes stimulated with 50 nM (*R*)-PAF *versus* unstimulated and 1 nM (*R*)-PAF-stimulated monocytes; monocytes stimulated with 1 nM (*R*)-PAF *versus* unstimulated monocytes. Δ——Δ, soluble fraction. $p = 0.00001$. The Student-Newman-Keuls test gave $p < 0.05$ for the following comparisons: unstimulated monocytes *versus* 50, 10, and 1 nM (*R*)-PAF-stimulated monocytes; monocytes stimulated with 1 nM (*R*)-PAF *versus* 50 and 10 nM (*R*)-PAF-stimulated monocytes. Results are the mean values ± S.D. of three experiments done in duplicate.

phorylation of CR1, adherent monocytes were labeled with ^{32}P, incubated with (*R*)-PAF (10 nM) and immunoprecipitated as described under "Experimental Procedures." Two bands of molecular mass approximating 190 and 220 kDa appeared labeled with ^{32}P in anti-CR1-immunoprecipitated membrane material from (*R*)-PAF-treated cells. The molecular masses correspond to those of allotypic forms of CR1 (31). Phosphorylation of CR1 was detectable in (*R*)-PAF-treated cells but not in untreated cells (Fig. 5).

Activation of PKC in Adherent Monocytes Stimulated with (R)-PAF—In resting adherent monocytes, 90–95% of total PKC activity eluted with 0.06–0.09 M NaCl from DEAE-52 was recovered in the soluble fraction (Fig. 6*A*), whereas 5–10% of the activity was found in the particulate fraction (not shown). A second peak of phospholipid-independent kinase activity was eluted with 0.13–0.17 M NaCl (Fig. 6*A*).

Exposure of adherent monocytes to 10 nM (*R*)-PAF for 3 min decreased cytosolic PKC activity to 15% of control value and increased PKC activity in the particulate fraction correspondingly (Fig. 6*B*). The PKC redistribution after addition of (*R*)-PAF application peaked after 3 min and returned to prestimulus level after 7 min (Fig. 6*B*). Thus, the kinetics of PKC activation was faster and more shortlived than the biological effect. The concentrations of (*R*)-PAF that enhanced phagocytosis remarkably stimulated translocation of PKC to the membrane compartment (Fig. 7). As shown in Table V, the specific receptor antagonists of (*R*)-PAF did not change the subcellular distribution of PKC activity in nonstimulated monocytes but almost completely prevented (*R*)-PAF-induced translocation of PKC activity. 100 nM (*S*)-PAF (a concentration 10 times higher than that of (*R*)-PAF) and

TABLE V

Specificity of (R)-PAF-induced PKC activation in adherent monocytes

Partially purified PKC was obtained from adherent monocytes stimulated as indicated for 3 min at 37 °C and assayed as described under "Experimental Procedures." Results are the mean values ± S.D. of three experiments done in triplicate.

Additions[a]	Net PKC activity	
	Soluble fraction	Particulate fraction
	pmol ^{32}P incorporated in 40 μg histone/min/10^7 cells	
1. None	636 ± 125	87 ± 36
2. (*R*)-PAF (10 nM)	121 ± 63	536 ± 141
3. (*S*)-PAF (100 nM)	518 ± 74	103 ± 71
4. Lyso-PAF (1 μM)	500 ± 87	106 ± 37
5. BN52021 (50 μM) + (*R*)-PAF 10 (nM)	696 ± 150	103 ± 84
6. BN52021 (50 μM)	631 ± 74	56 ± 81
7. CV3988 (50 μM) + (*R*)-PAF (10 nM)	601 ± 84	107 ± 64
8. CV3988 (50 μM)	580 ± 100	74 ± 23
	$p = 0.00001$[b]	$p = 0.00001$

[a] Adherent monocytes were incubated for 10 min at 37 °C with BN52021 or CV3988 before the addition of (*R*)-PAF or medium. Me_2SO used to dissolve BN52021 did not interfere with PKC activity (0.1% Me_2SO translocates 0.001 and 0% PKC activity after 3 and 5 min, respectively).

[b] For each column of data, *p* was calculated by two-way ANOVA. Whenever the ANOVA gave $p < 0.05$, the Student-Neuwman-Keuls test was applied for more specific comparisons. The following comparisons gave $P < 0.05$. Net PKC activity in soluble fraction: 5 *versus* 2, 1 *versus* 2, 6 *versus* 2, 7 *versus* 2, 8 *versus* 2, 3 *versus* 2, 4 *versus* 2; net PKC activity in particulate fraction: 2 *versus* 6, 2 *versus* 8, 2 *versus* 1, 2 *versus* 5, 2 *versus* 3, 2 *versus* 4, 2 *versus* 7.

1 μM lyso-PAF were ineffective.

Effect of PKC Inhibitors on Phagocytosis by Unstimulated and (R)-PAF-treated Adherent Monocytes—The effect of the PKC inhibitors H7 (32), palmitoylcarnitine (33), sphingosine (34), and staurosporin (35) on phagocytosis by unstimulated and (*R*)-PAF-stimulated monocytes is shown in Table VI. These compounds prevented the (*R*)-PAF-induced increase of phagocytosis of RBC^sC3b. However, only sphingosine and 10 μM staurosporin inhibited the phagocytosis of diamide-treated RBC^{hu} incubated in whole serum ($p < 0.05$), whereas H7 and palmitoylcarnitine decreased the phagocytosis but without statistical significance. All compounds did not modify the basal values of phagocytosis by unstimulated monocytes. PKC inhibitors did not affect the binding of RBC^sC3b to monocytes (not shown). The PKC inhibitors acted in a dose-dependent manner. The minimal concentrations of H7, palmitoylcarnitine, sphingosine, and staurosporin which maximally inhibited the effect of (*R*)-PAF were 50, 20, 20, and 10 μM, respectively. At these concentrations, the PKC inhibitors did not induce cell detachment and did not reduce cell viability (not shown).

TABLE VI

Effect of PKC inhibitors on phagocytosis by unstimulated and (R)-PAF-stimulated monocytes

Adherent monocytes (7×10^4) were incubated for 10 min at 37 °C with or without the indicated PKC inhibitors before the addition of 10 nM (*R*)-PAF. Phagocytosis was performed as described under "Experimental Procedures." Results are the mean values ± S.D. of four experiments done in triplicate.

Additions	Unstimulated monocytes		(*R*)-PAF-stimulated monocytes	
	Diamide-treated RBC^{hu}[a]	RBC^sC3b	Diamide-treated RBC^{hu}	RBC^sC3b
	ingested RBC/monocyte			
H7				
1. None	1.9 ± 0.6	0.7 ± 0.2	3.9 ± 1.6	5.6 ± 1.2
2. 5 μM	1.7 ± 0.9	0.5 ± 0.3	3.0 ± 0.7	3.8 ± 1.2
3. 50 μM	2.1 ± 0.3	0.9 ± 0.4	2.3 ± 0.5	1.2 ± 0.5
	$p = 0.0100$[b]	$p = 0.1934$	$p = 0.0556$	$p = 0.00001$
Palmitoylcarnitine				
2. 1 μM	1.7 ± 1.0	0.3 ± 0.4	4.1 ± 0.7	5.5 ± 0.3
3. 10 μM	1.6 ± 0.3	0.7 ± 0.9	3.6 ± 0.8	3.7 ± 0.2
4. 20 μM	1.8 ± 0.5	1.0 ± 0.5	1.9 ± 1.2	1.8 ± 0.4
	$p = 0.4608$	$p = 0.1410$	$p = 0.0583$	$p = 0.0008$
Sphingosine				
2. 1 μM	2.1 ± 0.9	0.2 ± 0.3	3.1 ± 1.3	4.7 ± 1.1
3. 10 μM	1.9 ± 0.5	0.4 ± 0.2	1.6 ± 0.7	3.2 ± 0.5
4. 20 μM	1.9 ± 0.8	0.8 ± 0.3	1.8 ± 1.1	1.9 ± 0.3
	$p = 0.3365$	$p = 0.4752$	$p = 0.0019$	$p = 0.00001$
Staurosporin				
2. 0.1 μM	1.8 ± 0.4	0.9 ± 0.2	4.2 ± 0.5	5.1 ± 1.2
3. 1 μM	2.1 ± 0.7	0.6 ± 0.4	3.1 ± 0.6	2.8 ± 0.3
4. 10 μM	2.4 ± 0.5	0.5 ± 0.2	2.1 ± 0.5	1.6 ± 0.2
	$p = 0.1431$	$p = 0.1074$	$p = 0.0014$	$p = 0.0007$

[a] Diamide-treated and untreated RBC^{hu} were incubated in whole serum as described under "Experimental Procedures." Me_2SO used to dissolve staurosporin did not interfere with phagocytosis (not shown).

[b] For each column of data, *p* was calculated by two-way ANOVA. Whenever the ANOVA gave $p < 0.05$, the Student-Newman-Keuls test was applied for more specific comparisons. The following comparisons gave $p < 0.05$. Phagocytosis of diamide-treated RBC^{hu} by (*R*)-PAF-stimulated monocytes incubated with sphingosine: 3 *versus* 2, 4 *versus* 1, 4 *versus* 2; phagocytosis of diamide-treated RBC^{hu} by (*R*)-PAF-stimulated monocytes incubated with staurosporin: 1 *versus* 4, 2 *versus* 4; phagocytosis of RBC^sC3b by (*R*)-PAF-stimulated monocytes incubated with H7: 1 *versus* 3; 1 *versus* 2, 2 *versus* 3; phagocytosis of RBC^sC3b by (*R*)-PAF-stimulated monocytes incubated with palmitoylcarnitine: 1 *versus* 4, 1 *versus* 3, 2 *versus* 4, 2 *versus* 3, 3 *versus* 4; phagocytosis of RBC^sC3b by (*R*)-PAF-stimulated monocytes incubated with sphingosine: 1 *versus* 4, 1 *versus* 3, 2 *versus* 4, 2 *versus* 3; phagocytosis of RBC^sC3b by (*R*)-PAF-stimulated monocytes incubated with staurosporin: 1 *versus* 4, 1 *versus* 3, 2 *versus* 4, 2 *versus* 3.

DISCUSSION

Oligomerization of band 3 in the human RBC membrane has recently been shown to trigger deposition of naturally occurring anti-band 3 antibodies and of C3 fragments (10–13). Oligomerization of band 3 and increasing binding of anti-band 3 antibody and C3 were noted in oxidatively stressed RBC^{hu}, in senescent RBC (11–13), and in glucose-6-phosphate dehydrogenase-deficient RBC isolated during fava bean hemolytic crisis.[2] All these types of damaged RBC were phagocytosed at a higher rate by human adherent monocytes and had shortened survival *in vivo* (11–13).

The present study demonstrates that recognition of the anti-band 3-C3 complex of the C3b receptor CR1 plays a crucial role in the removal process of damaged RBC by monocytes. Indeed, treatment of RBC in complement-inactivated serum and blocking of CR1 with anti-CR1 mAb abrogated phagocytosis of diamide-treated RBC incubated in whole serum. We have utilized RBC^{hu} treated with 20 μM diamide as phagocytic targets for the following reasons. (*a*) Diamide reacts within seconds with reduced thiols, mainly GSH, to produce sulfides (36). The reduction of S-S bonds generated within the membrane is probably slow since GSH, the actual reductant, is not permeant (37). (*b*) The diamide concentration used here did not measurably modify GSH levels, intermediary metabolism, calcium homeostasis, and

[2] H. Lutz, F. Turrini, and P. Arese, in preparation.

RBC^{hu} deformability, as assessed by ektacytometry (not shown). (c) The "diamide model" bears strong analogies to naturally senescent RBC or to pathologically altered RBC such as glucose-6-phosphate dehydrogenase-deficient RBC^{hu}.[3] The involvement of band 3 exposure in the removal of aged RBC ("senescent epitope") was described by Kay (38, 39). Denatured hemoglobin or Heinz bodies, commonly observed in sickle cell anemia, were found to induce pronounced clustering of band 3 (40, 41).

Relatively few CR1 molecules are expressed on the surface of resting circulating monocytes, but their number increases rapidly following exposure to chemotactic factors (42) or to other stimuli including phorbol esters (5, 43), diacylglycerols (44), calcium ionophore A23187 (45), and tumor necrosis factor (46). The rapid increase in the number of surface receptors and the insensitivity of the process to inhibitors of protein synthesis point to the translocation of preformed receptor from an intracellular pool to the cell surface. In addition to inducing alterations in the membrane expression of the receptor, phorbol esters activate CR1 on monocytes providing the receptor with the ability to mediate phagocytosis of C3b-coated targets (5, 14). Treatment with phorbol esters induces phosphorylation of CR1 in phagocytic cells suggesting that acquisition of phagocytic capability on CR1 on monocytes is correlated with PKC-dependent phosphorylation of the receptor (14).

PAF, a potent phospholipid autacoid, is involved in inflammatory processes (15, 16). The effects of PAF are mediated by binding to high affinity binding sites on target cells that trigger activation of phospholipase C, release of diacyglycerol, increase in cytoplasmic Ca^{2+} (15, 16), and stimulation of PKC (47, 48). Phosphorylation of several protein substrates consequent to PKC activation has been described in platelets stimulated with PAF at nanomolar concentrations (49). PAF has also been shown to stimulate expression of CR1 on human neutrophils (50). In the present work, we have been unable to demonstrate increased expression of CR1 by adherent monocytes in the presence of (R)-PAF. These results may depend on the fact that both monocyte purification and monocyte adhesion are sufficient to enhance membrane expression of the receptor.

We show here that nanomolar concentrations of (R)-PAF greatly enhanced CR1-dependent phagocytosis of diamide-treated RBC^{hu} incubated in whole serum by adherent monocytes. In addition, (R)-PAF rendered adherent monocytes capable of ingesting $RBC^{s}C3b$. (R)-PAF induced the fast translocation of PKC from cytosol to the membrane compartment and phosphorylation of CR1. Concentrations of (R)-PAF that were active in triggering CR1-dependent phagocytosis were also active in inducing PKC translocation and phosphorylation of the receptor. The effects reported here were specific for (R)-PAF since lyso-PAF and the biologically inert (S)-PAF were inactive. Furthermore, two specific PAF receptor antagonists almost completely abrogated the effects of (R)-PAF on phagocytosis and PKC translocation. Peak activation of PKC translocation occurs 3–5 min after (R)-PAF addition. Five minutes after (R)-PAF stimulation, phagocytosis was already increased by 75%, and maximal stimulation was attained after 10 min. At that time point, membrane-associated PKC was declining, but CR1 was phosphorylated. Thus, the time course of the three events—stimulation of PKC translocation, CR1 phosphorylation, and enhancement of phagocytosis—is compatible with a cause-and-effect relationship between the temporally first effect and the last one.

Specific PKC inhibitors (H7, palmitoylcarnitine, sphingosine, and staurosporin) blocked the enhancement of phagocytosis induced by PAF without affecting the basal phagocytic activity of the monocytes, as has also been demonstrated in neutrophils (51).

PAF concentrations found effective in this work are within the range of concentrations generated during inflammatory processes (15, 16) and may clarify hitherto unexplained massive RBC removal in cases in which oxidant damage to RBC and inflammation coexist. An example is offered by hemolytic crises in glucose-6-phosphate dehydrogenase-deficient individuals during infection or sepsis but in the absence of other triggers such as fava beans or oxidant drugs (52). Another example is the massive RBC removal that occurs in sickle cell anemia concomitant with infections (53). (R)-PAF-elicited modulation of CR1-dependent phagocytosis may be expected to play a role in a variety of other disorders in which bacterial or parasitic pathogens interact with human monocytes via CR1 (54, 55). Triggering by PAF of PKC translocation, phosphorylation of CR1, and stimulation of the receptor to an active state capable of mediating phagocytosis represents a novel pathway by which PAF modulates inflammatory reactions and host defense mechanisms against infection.

[3] H. Lutz, F. Turrini, S. Fasler, P. Stammler, and P. Arese, in preparation.

REFERENCES

1. Hogg, N. (1988) *Immunol. Today (Amst.)* **9,** 185–187
2. Fearon, D. T., and Wong, W. W. (1983) *Annu. Rev. Immunol.* **1,** 243–271
3. Myones, B. L., Daizell, J. G., Hogg, N., and Ross, G. D. (1988) *J. Clin. Invest.* **82,** 640–651
4. Griffin, J. A., and Griffin, F. M., Jr. (1979) *J. Exp. Med.* **150,** 653–675
5. Wright, S. D., and Silverstein, S. C. (1982) *J. Exp. Med.* **156,** 1149–1164
6. Pommier, C. G., Inada, S., Fries, L. F., Takahashi, T., Frank, M. M., and Brown, E. J. (1983) *J. Exp. Med.* **157,** 1844–1854
7. Bohnsack, J. F., Kleinman, H. K., Takahashi, T., O'Shea, J., Jr., and Brown, E. J. (1985) *J. Exp. Med.* **161,** 912–923
8. Wright, S. D., Craigmyle, L. S., and Silverstein, S. C. (1983) *J. Exp. Med.* **158,** 1338–1343
9. Clark, M. R., and Shohet, S. B. (1985) *Clin. Haematol.* **14,** 223–257
10. Lutz, H. U. (1987) *Biomed. Biochim. Acta* **46,** 65–71
11. Lutz, H. U., Bussolino, F., Flepp, R., Fasler, S., Stammler, P., Kazatchkine, M. D., and Arese, P. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 7368–7372
12. Lutz, H. U., Fasler, S., Stammler, P., Bussolino, F., and Arese, P. (1988) *Blood Cells* **14,** 175–195
13. Lutz, H. U., Stammler, P., Furter, C., and Fasler, S. (1987) *Schweiz. Med. Wochenschr.* **117,** 1821–1824
14. Changelian, P. S., and Fearon, D. T. (1986) *J. Exp. Med.* **163,** 101–115
15. Braquet, P., Touqui, L., Shen, T. Y., and Vargaftig, B. B. (1987) *Pharmacol. Rev.* **39,** 97–145
16. Hanahan, D. J. (1986) *Annu. Rev. Biochem.* **55,** 483–509
17. Yasaka, T., Boxer, L. A., and Bahner, R. L. (1982) *J. Immunol.* **128,** 1939–1944
18. Conrad, G. W., and Rink, T. J. (1986) *J. Cell Biol.* **103,** 439–450
19. Huang, S. J., Monk, P. N., Downes, C. P., and Whetton, A. D. (1988) *Biochem. J.* **249,** 839–845
20. Camussi, G., Bussolino, F., Tetta, C., Piacibello, W., and Aglietta, M. (1983) *Int. Arch. Allergy Appl. Immunol.* **70,** 245–252
21. Albert, D. H., and Snyder, F. (1983) *J. Biol. Chem.* **258,** 97–102
22. Cook, J., Fischer, E., Boucheix, C., Mirsrahi, M., Jouvin, M. H., Weiss, L., Jack, R. M., and Kazatchkine, M. D. (1985) *Mol. Immunol.* **22,** 531–539
23. Fearon, D. T. (1980) *J. Exp. Med.* **153,** 20–30
24. Steinbuch, M., and Audran, R. (1969) *Arch. Biochem. Biophys.* **134,** 279–284
25. Fischer, E., and Kazatchkine, M. D. (1983) *J. Immunol.* **130,** 2821–2824

26. Bussolino, F., Turrini, F., and Arese, P. (1987) *Br. J. Haematol.* **66,** 271–274
27. Boyum, A. (1969) *Scand. J. Clin. Lab. Invest.* **21,** (Suppl. 97), 77–89
28. Labarca, C., and Paigen, K. (1980) *Anal. Biochem.* **102,** 344–352
29. Kikkawa, U., Takai, Y., Minakuchi, R., Inohara, S., and Nishizuka, Y. (1982) *J. Biol. Chem.* **257,** 13341–13348
30. Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U., and Nishizuka, Y. (1982) *J. Biol. Chem.* **257,** 7847–7851
31. Dykman, T. R., Hatch, J. A., and Atkinson, J. P. (1984) *J. Exp. Med.* **159,** 691–698
32. Hidaka, H., Inagaki, M., Kawamoto, S., and Sasaki, Y. (1984) *Biochemistry* **23,** 5036–5041
33. Ganong, B. R., Loomis, C. R., Hannu, Y. A., and Bell, R. M. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 1184–1188
34. Hannu, Y. A., Loomis, C. R., Merril, A. H., Jr., and Bell, R. M. (1986) *J. Biol. Chem.* **261,** 12604–12609
35. Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M., and Tomita, F. (1986) *Biochem. Biophys. Res. Commun.* **135,** 397–402
36. Kosower, N. S., Kosower, E. M., Wertheim, B., and Correa, W. S. (1969) *Biochem. Biophys. Res. Commun.* **37,** 593–596
37. Harris, J. W. (1979) *Pharmacol. Ther.* **7,** 375–391
38. Kay, M. M. B. (1975) *Proc. Natl. Acad. Sci. U. S. A.* **72,** 3521–3525
39. Kay, M. M. B. (1978) *J. Supramol. Struct.* **9,** 555–567
40. Low, P. S., Waugh, S. M., Zinke, K., and Drenckhahn, D. (1985) *Science* **227,** 531–533
41. Waugh, S. M., Willardson, B. M., Kannan, R., Labotka, R. J., and Low, P. S. (1986) *J. Clin. Invest.* **78,** 1155–1160
42. Fearon, D. T., and Collins, L. A. (1983) *J. Immunol.* **130,** 370–375
43. Wright, S. D., and Meyer, B. C. (1986) *J. Immunol.* **136,** 1759–1764
44. O'Shea, J. J., Siwik, S. A., Gaither, T. A., and Frank, M. M. (1985) *J. Immunol.* **135,** 3381–3387
45. Berger, M., Birx, D. L., Wetzler, E. M., O'Shea, J. J., Brown, E. J., and Cross, A. S. (1985) *J. Immunol.* **135,** 1342–1348
46. Berger, M., Wetzler, E. M., and Wallis, R. S. (1988) *Blood* **71,** 151–158
47. Gay, J. C., and Stitt, E. S. (1988) *Blood* **71,** 159–165
48. O'Flaherty, J. T., and Nishihira, J. (1987) *J. Immunol.* **138,** 1889–1895
49. Sugatani, J., and Hanahan, D. J. (1986) *Arch. Biochem. Biophys.* **246,** 855–864
50. Shalit, M., Von Allmen, C., Atkins, P. C., and Zweiman, B. (1987) *Clin. Res.* **35,** 257 (abstr.)
51. Wilson, E., Olcott, M. C., Bell, R. M., Merril, A. H., Jr., and Lambeth, J. D. (1986) *J. Biol. Chem.* **261,** 12616–12623
52. Dacie, J. (1985) *The Haemolytic Anaemias,* Vol. 1, Part 1, pp. 378–379, Churchill Livingstone, Edinburgh
53. Vichinski, E., and Lubin, B. H. (1987) in *Hematology/Oncology of North America: Pediatric Hematology* (Oski, F. A., ed) Vol. 1, pp. 483–501, W. B. Saunders, Philadelphia
54. Payne, N. R., and Horwitz, M. A. (1987) *J. Exp. Med.* **166,** 1377–1389
55. Mosser, D. M., and Edelson, P. J. (1987) *Nature* **327,** 329–331