

Chloride Ion Efflux Regulates Adherence, Spreading, and Respiratory Burst of Neutrophils Stimulated by Tumor Necrosis Factor- α (TNF) on Biologic Surfaces

Renzo Menegazzi, Sara Busetto, Pietro Dri, Rita Cramer, and Pierluigi Patriarca

Istituto di Patologia Generale, Università di Trieste, Trieste, Italy

Abstract. Chloride ion efflux is an early event occurring after exposure of neutrophilic polymorphonuclear leukocytes (PMN) in suspension to several agonists, including cytokines such as tumor necrosis factor- α (TNF) and granulocyte/macrophage-colony stimulating factor (Shimizu, Y., R.H. Daniels, M.A. Elmore, M.J. Finnen, M.E. Hill, and J.M. Lackie. 1993. *Biochem. Pharmacol.* 9:1743–1751). We have studied TNF-induced Cl^- movements in PMN residing on fibronectin (FN) (FN-PMN) and their relationships to adherence, spreading, and activation of the respiratory burst. Occupancy of the TNF-R55 and engagement of $\beta 2$ integrins cosignaled for an early, marked, and prolonged Cl^- efflux that was accompanied by a fall in intracellular chloride levels (Cl^-_i). A possible causal relationship

between Cl^- efflux, adherence, and respiratory burst was first suggested by kinetic studies, showing that TNF-induced Cl^- efflux preceded both the adhesive and metabolic response, and was then confirmed by inhibition of all three responses by pretreating PMN with inhibitors of Cl^- efflux, such as ethacrynic acid. Moreover, Cl^- efflux induced by means other than TNF treatment, i.e., by using Cl^- -free media, was followed by increased adherence, spreading, and metabolic activation, thus mimicking TNF effects. These studies provide the first evidence that a drastic decrease of Cl^-_i in FN-PMN may represent an essential step in the cascade of events leading to activation of proadhesive molecules, reorganization of the cytoskeleton network, and assembly of the O_2^- -forming NADPH oxidase.

NEUTROPHILIC polymorphonuclear leukocytes (PMN)¹ respond to both particulate and soluble stimuli with a vigorous respiratory burst. This leads to the release of toxic oxygen molecules that contribute to both the PMN microbicidal activity and the tissue inflammatory damage. Among the physiologically relevant soluble stimuli, cytokines such as tumor necrosis factor- α (TNF), granulocyte/macrophage-colony stimulating factor, and granulocyte-colony stimulating factor are peculiar, since they activate the respiratory burst only in PMN residing on biologic surfaces, e.g., on proteins of the extra-

cellular matrix immobilized on a solid support, but not in PMN in suspension (34, 38, 39, 40).

TNF is the most extensively studied in this respect. It has been shown that PMN exposed to TNF on several biologic surfaces, e.g., FBS, fibronectin (FN), and fibrinogen (FBG), adhere, spread, and activate their oxidative metabolism (18, 34, 37, 41).

Several lines of evidence indicate that this series of responses is cosignaled by ligation of TNF receptors (TNF-R), more specifically the 55-kD TNF-R (TNF-R55) (34), and binding of activated proadhesive molecules to receptors on the biologic substrates. CD11/CD18 ($\beta 2$) integrins have been shown to be relevant in this respect, since: (a) PMN of leukocyte adhesion deficiency patients, which lack $\beta 2$ integrin expression (29), fail to respond to TNF with a metabolic burst (38), and (b) anti- $\beta 2$ mAbs inhibit both adherence and respiratory burst of PMN exposed to TNF on biologic surfaces (32, 34, 37, 38).

The relative role of TNF- and $\beta 2$ integrin-derived intracellular signals has not been clearly defined. It has been hypothesized that engagement of the TNF receptor leads to a modification, often called "activation," of the $\beta 2$ integrins, which results in an increased affinity for the appropriate surface ligand and in an adhesive PMN response

Address all correspondence to R. Menegazzi, Istituto di Patologia Generale, Università di Trieste, via A. Fleming, 22, 34127 Trieste, Italy. Tel.: (39) 40 572012. Fax: (39) 40 567862.

1. *Abbreviations used in this paper:* 9-AC, anthracene-9-carboxylic acid; CHC, α -cyano-4-hydroxy-cinnamic acid; Cl^-_i , intracellular chloride content; DIDS, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid; EA, ethacrynic acid; FBG, fibrinogen; FMLP, *N*-formyl-methionyl-leucyl-phenylalanine; FN, fibronectin; FN-PMN, PMN residing on FN-coated surfaces; G-buffer, glucuronate-containing buffer; MA, *o*-[(3-hydroxymercuri-2-methoxypropyl)carbamoyl] phenoxyacetic acid; O_2^- , superoxide anion; PMN, neutrophilic polymorphonuclear leukocytes; s-PMN, PMN in suspension; poly(HEMA), poly(2-hydroxyethyl methacrylate); TNF, tumor necrosis factor- α ; TNF-R, TNF receptor.

(33, 34, 37, 46, 59). Once adherent, the cells would rely on the signaling ability of $\beta 2$ integrins to initiate the process of spreading with the ongoing activation of the respiratory burst (5, 37, 39, 41).

The nature of the intracellular signals involved in TNF-induced activation of PMN oxidative metabolism on biologic surfaces has been a matter of recent studies. The release of toxic oxygen molecules by TNF-stimulated PMN has been shown to be independent of a pertussis toxin-sensitive GTP-binding protein (31), weakly sensitive to protein kinase C inhibitors (31), and strongly impaired by drugs that interfere with the assembly of the cytoskeleton network (31, 34, 37, 39). Furthermore, it is not accompanied by hydrolysis of phosphoinositides nor by the release of arachidonic acid (31). It has been also shown that occupation of TNF receptors and engagement of CD18 ($\beta 2$) integrins interact synergistically to promote a sustained fall in cAMP in PMN plated on FBS- or FBG-coated surfaces (37). This drop in cAMP is permissive for TNF-induced spreading and metabolic activation, since cAMP-elevating agents prevent both responses. Also, tyrosine phosphorylation has been demonstrated to be essential for PMN spreading and metabolic activation induced by TNF, since inhibitors of tyrosine kinases prevent spreading and metabolic activation of these cells on FBS- and FBG-coated surfaces (18, 19, 32). The participation of $\beta 2$ integrin-derived signals in mediating protein tyrosine phosphorylation is formally proved by the observation that TNF does not trigger this response in leukocyte adhesion deficiency PMN (6, 19).

Studies on other possible intracellular signals evoked in PMN by TNF include the analysis of inorganic ion movements. Elevations in cytosolic free Ca^{2+} levels are still a matter of debate. In fact, while some authors obtained evidence for TNF-induced intracellular Ca^{2+} changes (44, 48), others did not (42, 49). Other groups demonstrated that TNF causes a decrease of intracellular H^+ concentration that leads to cytoplasmic alkalinization (49, 64).

Recently, attention has been focused on chloride ion movements in TNF-stimulated PMN (48, 49). The intracellular Cl^- concentration of resting PMN is unusually high, being fourfold higher than that predicted on the basis of Nernst equation (3, 51). It has been shown that TNF (49), as well as other soluble stimuli (36, 49), causes an efflux of chloride ions from PMN in suspension, with the concomitant decrease of the intracellular chloride content (Cl^-_i). The relevance of such a change to effector functions of PMN, e.g., adherence, spreading, and activation of the metabolic burst, has not been investigated.

In this paper, we show that TNF-stimulated PMN residing on FN-coated surfaces (FN-PMN) exhibit a massive Cl^- efflux accompanied by a concomitant decrease of Cl^-_i ; that precedes adhesion, spreading, and activation of the respiratory burst. Inhibition of Cl^- efflux also inhibits adhesion, spreading, and activation of the burst, whereas induction of Cl^- fluxes by means other than TNF treatment (i.e., the use of Cl^- -free media) also causes PMN to adhere, spread, and mount a respiratory burst on FN. We therefore suggest that Cl^- efflux plays a role in the elicitation of these three functions of FN-PMN. We also show that the TNF-dependent cascade of Cl^- efflux, adhesion, spreading, and respiratory burst of FN-PMN is initiated by occupancy of the TNF-R55 receptor.

Materials and Methods

Reagents

D-glucuronic acid sodium salt, D-gluconic acid sodium salt, L-glutamic acid sodium salt, cytochrome c (type VI, from horse heart), anthracene-9-carboxylic acid (9-AC), [2,3-dichloro-4-(2-methylene-butyl)phenoxy]acetic acid (ethacrynic acid [EA]), *o*-[(3-hydroxymercuri-2-methoxypropyl)carbamoyl]phenoxyacetic acid; mersalyl acid [MA] 5-[aminosulfonyl]-4-chloro-2-[(2-furanylmethyl)amino]benzoic acid (furosemide), (3-[aminosulfonyl]-5-[butylamino]-4-phenoxybenzoic acid) (bumetanide), (α -cyano-4-hydroxy-cinnamic acid (CHC), 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS), *N*-formyl-methionyl-leucyl-phenylalanine (FMLP), PMA, and Triton X-100 were obtained from Sigma Chemical Co. (St. Louis, MO). Cytochalasin B and 3,3',5,5'-tetramethylbenzidine were obtained from Serva Feinbiochemica (Heidelberg, Germany). Percoll was obtained from Pharmacia (Uppsala, Sweden). Fibronectin was purified from human plasma by affinity chromatography on gelatin, according to Ruoslahti et al. (45). Human recombinant TNF, produced in the yeast *Pichia pastoris*, was obtained from Bissendorf Biochemicals GmbH (Hannover, Germany). Na^{36}Cl (sp act 14 $\mu\text{Ci/g Cl}^-$) was purchased from Amersham Intl. (Amersham, UK).

Antibodies

Purified mouse IgG1 mAb htr-9 and mAb utr-1, specific for the 55-kD TNF receptor (TNF-R55) and the 75-kD TNF-R (TNF-R75), respectively (8), were a generous gift of Dr. M. Brockhaus (Hoffmann-La Roche, Basel, Switzerland). Purified mouse IgG2a mAb H398, directed against the p55 TNF-R, was prepared as previously described (20, 58) and kindly donated by Dr. P. Scheurich (Institute of Cell Biology and Immunology, University of Stuttgart, Germany). mAb 60.3, a murine IgG2a that recognizes the CD18 subunit (common β chain) of the CD11/CD18 antigen complex (4), was kindly provided by Dr. J.M. Harlan (Washington University, Seattle, WA).

PMN Isolation

Peripheral blood anticoagulated with EDTA was obtained from blood donors or from laboratory personnel. PMN were isolated according to the method described by Metcalf et al. (35), with slight modifications. Briefly, 4 ml of anticoagulated fresh blood was layered onto a discontinuous gradient of 62 and 75% Percoll in PBS and centrifuged at 200 g for 10 min and then at 400 g for an additional 15 min. The neutrophil-containing layer was collected at the interface between the 62 and 75% Percoll and washed once in Hepes buffer (145 mM NaCl, 5 mM KCl, 1 mM MgCl_2 , 1 mM CaCl_2 , 5 mM glucose, 5 mM Hepes buffer, pH 7.4, and 0.2% BSA) without Ca^{2+} and Mg^{2+} . After a 10-s hypotonic lysis of contaminating erythrocytes, isolated PMN were washed again in Ca^{2+} and Mg^{2+} -free Hepes buffer and resuspended at the desired concentration in the same medium. The resulting cell population contained 95–97% neutrophils, 2–3% eosinophils, and 1–2% mononuclear cells. Unless otherwise stated, CaCl_2 and MgCl_2 were added to the cell suspension just before the start of the functional assays.

Preparation of Surfaces

The coating of flat-bottomed microtiter plate-wells (MaxiSorp Immuno microwell plates, 442404; Nunc, Roskilde, Denmark) and of 6-well tissue-culture plates (Multidish 6-wells, 152795; Nunc) with FN was performed as previously described (15). Briefly, 50 or 750 μl of 20 $\mu\text{g/ml}$ FN in PBS was deposited into replicate wells of microtiter or 6-well tissue-culture plates, respectively. The plates were then left at 37°C for 1 to 2 h in a humidified incubator. Just before use, the wells were washed three times with PBS.

Measurements of $^{36}\text{Cl}^-$ Efflux from PMN in Suspension and Residing on FN-coated Surfaces

PMN were preloaded with $^{36}\text{Cl}^-$ following the method described by Simchowicz and De Weer (51). Briefly, PMN were suspended at $10\text{--}25 \times 10^6$ cells per ml in Hepes buffer and incubated with $^{36}\text{Cl}^-$ (3.0 $\mu\text{Ci/ml}$) for 2 h at 37°C in a shaking water bath to equilibrate $^{36}\text{Cl}^-$ between the intracellular and extracellular compartment (51). For the $^{36}\text{Cl}^-$ efflux studies, the cells were then washed twice with prewarmed unlabeled buffer to remove

the tracer and suspended in the same buffer at 2.5×10^6 cells per ml. Measurements of $^{36}\text{Cl}^-$ efflux from PMN in suspension were performed by incubating the cells in poly(2-hydroxyethyl methacrylate) (poly[HEMA]) test tubes with or without 10 ng/ml TNF. At the desired times, 800- μl aliquots of the cell suspension were collected from duplicate tubes and centrifuged for 30 s at 12,000 g. Then, 750- μl aliquots of the supernatants were withdrawn, and their radioactivity was counted by liquid scintillation counting in a β counter (LS6000TA; Beckman Instruments, Inc., Fullerton, CA). Measurements of $^{36}\text{Cl}^-$ efflux from PMN residing on FN-coated surfaces were performed by incubating the cells in 6-well tissue-culture plates. 800- μl aliquots of PMN loaded with $^{36}\text{Cl}^-$ were transferred to duplicate wells, and the incubation was then started by adding 8 μl of 1 $\mu\text{g}/\text{ml}$ TNF (10 ng/ml final concentration). At the desired times, the incubation medium was collected from the wells and spun for 30 s at 12,000 g to pellet detached cells. 750- μl aliquots of the supernatants were then dispersed in the scintillation liquid and counted as described above. To assay the effect of Cl^- transport inhibitors, PMN were preincubated in suspension with the required compound for 10 min at 37°C . The percentage of efflux was calculated as follows: $[(\text{cpm in the supernatant of time}_x \text{ sample}) - (\text{cpm in the supernatant of time}_0 \text{ sample})] / [(\text{total cpm of cell suspension}) - (\text{cpm of } t_0 \text{ supernatant})] \times 100$.

Measurements of Intracellular $^{36}\text{Cl}^-$ Content ($^{36}\text{Cl}^-$)_i of PMN in Suspension and Residing on FN-coated Surfaces

To determine the net movement of $^{36}\text{Cl}^-$ in PMN, the changes in $^{36}\text{Cl}^-$ _i were measured. PMN suspended at 2.5×10^6 cells per ml in Hepes buffer were loaded with $^{36}\text{Cl}^-$ as described in the previous paragraph and used without washing them free of the tracer. The assay with PMN in suspension was performed by incubating the cells in poly(HEMA) test tubes. At the desired times, 800- μl aliquots of the PMN suspension were diluted in prewarmed PBS and exhaustively washed. After the last washing, the bottom of the tubes was cut off, and the cell pellet-associated radioactivity was measured as described above. Background value of $^{36}\text{Cl}^-$ eventually trapped between intercellular spaces in the cell pellet was measured by centrifuging an aliquot of the cell suspension immediately after the addition of Na^{36}Cl for the loading procedure. This radioactivity was found to account for <2% of the total radioactivity of the cell pellet after 2 h of loading with the tracer and, therefore, it was neglected in the calculations of $^{36}\text{Cl}^-$ _i. The $^{36}\text{Cl}^-$ that remained associated to the cell pellet was expressed as follows: $(\text{cpm in the cell pellet of time}_x \text{ sample}) / (\text{cpm in the cell pellet of } t_0 \text{ sample}) \times 100$. Measurements of $^{36}\text{Cl}^-$ _i of PMN residing on FN were made in cells incubated in 6-well tissue-culture plates coated with FN as described above. On completion of the incubation, the plate was spun at 400 g for 10 min, and the supernatants were discarded. After exhaustively washing the wells with prewarmed PBS, the cell pellet was harvested in 1% Triton X-100, and the associated radioactivity was counted as described above. Background value of $^{36}\text{Cl}^-$ associated with the cells pelleted at the well bottom was measured with an aliquot of the cell suspension collected immediately after addition of Na^{36}Cl for the loading procedure. The $^{36}\text{Cl}^-$ that remained associated to PMN was calculated as follows: $[(\text{cpm in the pellet of time}_x \text{ sample}) - (\text{background cpm})] / [(\text{cpm in the pellet of time}_0 \text{ sample}) - (\text{background cpm})] \times 100$.

Assay of O_2^- Production

Production of superoxide anion (O_2^-) was measured by means of the superoxide dismutase-inhibitable cytochrome c reduction adapted for use in a microplate reader (Multiskan MCC/340; Labsystems Oy, Helsinki, Finland), as described in detail elsewhere (15). Briefly, 50 μl of PMN suspension (1.5×10^6 cells per ml) was added to the wells containing, in a 100- μl vol, 0.12 mM cytochrome c, TNF, or other stimuli at a concentration 3.0 times as high as the desired final concentration. Both the cell suspension and the plate were prewarmed for 5 min at 37°C . At the desired times, the plate was read at 550 and 540 nm. The amount of reduced cytochrome c was calculated from the absorbance difference between 550 and 540 nm, using as a standard an absorbance of 0.037 OD units for 1 mmol of reduced cytochrome c.

Assay of Adherence

Adherence assay was performed as previously described (16), with the exception of a modification of the washing procedure to remove nonadherent PMN. Briefly, on completion of the incubation, the wells were filled

with PBS, sealed up with strings of eight caps (cap band-8, MS22705; Micronic, Lelystad, The Netherlands), and then the plate was centrifuged upside down at 200 g for 5 min. After centrifugation, the strings were removed, the wells were flicked empty, and the quantitation of adherent PMN was performed by an enzymatic assay based on the measurement of myeloperoxidase activity (14). Unless otherwise stated, the assay of adherence was performed in the same wells as O_2^- release assays after the last reading for O_2^- production was taken.

Assessment of Cell Spreading

2.5×10^5 PMN in 150 μl were added to 16-mm-diam wells (NUNC, Inc., Naperville, IL) containing 13-mm-diam tissue-culture dishes (Thermanox; NUNC, Inc.) coated with FN as described above. On completion of the incubation procedures, the cells were fixed by adding an equal volume of 1% glutaraldehyde in PBS. The dishes were then turned upside down on a drop of PBS and observed under phase contrast with a microscope (Leitz Biomed; Leica Mikroskopie & Systeme GmbH, Wetzlar, Germany).

Scanning Electron Microscopy

2.5×10^5 PMN were deposited onto 13-mm-round tissue-culture dishes (Thermanox, NUNC, Inc.), coated with FN as described above. After incubation, nonadherent cells were removed by gently aspirating the incubation medium. The neutrophils that remained adherent to the surfaces were fixed with 2% glutaraldehyde, dehydrated in graded ethanols (30–100%), and then processed by conventional techniques for scanning EM.

Results

Chloride Movements in TNF-stimulated PMN

Chloride movements in FN-PMN or in suspension (s-PMN) were monitored by assaying $^{36}\text{Cl}^-$ efflux from and $^{36}\text{Cl}^-$ _i of cells preloaded with $^{36}\text{Cl}^-$ by incubation in a $^{36}\text{Cl}^-$ -containing medium. To assay $^{36}\text{Cl}^-$ efflux, the cells were washed free of the hot loading medium that was replaced with cold medium. TNF, or the appropriate control solution, was then added, and, at the end of the experiment, the $^{36}\text{Cl}^-$ released in the extracellular compartment was measured. To assay $^{36}\text{Cl}^-$ _i, which reflects net Cl^- movements, TNF, or the appropriate control solution, was added to $^{36}\text{Cl}^-$ -loaded cells maintained in the hot loading medium. At the end of the experiment, the cells were exhaustively washed free of the radioactive buffer, and the cell-associated $^{36}\text{Cl}^-$ was measured. The results, shown in Table I, were as follows:

(a) Resting s-PMN showed a 20% $^{36}\text{Cl}^-$ efflux after 20 min incubation, which did not appreciably vary in the next 20 min. The finding that this efflux was not paralleled by an equivalent drop in $^{36}\text{Cl}^-$ _i indicates that this does not reflect a net $^{36}\text{Cl}^-$ outflow, but rather is a consequence of Cl^-/Cl^- self-exchange.

(b) Addition of TNF to s-PMN induced a net $^{36}\text{Cl}^-$ efflux, as indicated by the concomitant decrease in $^{36}\text{Cl}^-$ _i by 20 min. The loss of $^{36}\text{Cl}^-$ _i was at least in part regained afterwards.

(c) Resting FN-PMN exhibited a net $^{36}\text{Cl}^-$ efflux, accompanied by a corresponding drop in $^{36}\text{Cl}^-$ _i, that was virtually complete within 20 min. The anti- $\beta 2$ integrin mAb 60.3 (10 $\mu\text{g}/\text{ml}$ final concentration), which inhibited the basal PMN adhesion by $\sim 30\%$ ($18.1 \pm 2.8\%$ adherence in the presence of mAb 60.3 vs $25.8 \pm 3.2\%$ control adherence, mean \pm SEM, $n = 3$), had a minor inhibitory effect on $^{36}\text{Cl}^-$ efflux from unstimulated FN-PMN ($34.9 \pm 3.8\%$ $^{36}\text{Cl}^-$ efflux in the presence of mAb 60.3 vs $39.1 \pm 2.2\%$ control $^{36}\text{Cl}^-$ efflux, mean \pm SEM, $n = 3$). This suggests

Table I. Chloride Movements in PMN in Suspension (s-PMN) or Residing on FN-coated Surfaces (FN-PMN)

	$^{36}\text{Cl}^-$ efflux (percentage of release)		$^{36}\text{Cl}^-_i$ (percentage of t_0)	
	s-PMN	FN-PMN	s-PMN	FN-PMN
	%		%	
rest _{20 min}	19.9 ± 1.6	35.8 ± 1.5	96.7 ± 1.3	75.8 ± 2.2
rest _{40 min}	21.0 ± 2.3	37.8 ± 4.0	91.0 ± 1.2	66.0 ± 2.0
TNF _{20 min}	54.4 ± 3.9	72.9 ± 1.9*	66.2 ± 2.3	26.5 ± 3.8
TNF _{40 min}	61.6 ± 3.0	84.5 ± 3.2*	75.3 ± 3.2	7.5 ± 2.8

Loading of PMN with $^{36}\text{Cl}^-$ was performed by incubating the cells for 2 h at 37°C with 3.0 $\mu\text{Ci/ml}$ Na^{36}Cl . To assay $^{36}\text{Cl}^-$ efflux, the cells were subjected to the experimental procedures after washing them free of the tracer. To assay $^{36}\text{Cl}^-_i$, PMN were used without washing. The cells were incubated at 37°C either in suspension in poly (HEMA) test tubes (s-PMN) or in tissue-culture plate-wells coated with FN (FN-PMN). To measure $^{36}\text{Cl}^-$ efflux, aliquots of the supernatants were collected at stated times from both the tubes and the wells and counted for radioactivity. Changes in $^{36}\text{Cl}^-_i$ in s-PMN and in FN-PMN were estimated by counting the cell pellet-associated radioactivity after the washing steps. Data are expressed as described in detail in Materials and Methods and represent the means ± SEM of three to five experiments (in duplicate).

*The differences between TNF-stimulated $^{36}\text{Cl}^-$ efflux of s-PMN and FN-PMN at 20 and 40 min were statistically significant ($P < 0.02$), as calculated by two-tailed t test on paired data.

that the $\beta 2$ -independent adhesion of PMN to FN is sufficient, per se, to induce a net outward Cl^- movement.

(d) Addition of TNF to FN-PMN drastically increased both $^{36}\text{Cl}^-$ efflux and drop in $^{36}\text{Cl}^-_i$. After 20 min, >70% of the intracellular tracer was lost. At variance with resting FN-PMN, this $^{36}\text{Cl}^-$ efflux continued afterwards and reached ~85% in the next 20 min. There was no evidence of a tendency to regain the lost intracellular $^{36}\text{Cl}^-$, suggesting that adhesion to FN counteracts the chloride "regain mechanism" operating in s-PMN. In the presence of mAb 60.3, which inhibited by ~60% TNF-induced adherence to FN ($25.8 \pm 4.3\%$ vs $62.8 \pm 3.7\%$ adherence, mean ± SEM, $n = 3$), the chloride efflux was markedly inhibited ($49.8 \pm 3.5\%$ vs $81.9 \pm 4.5\%$ $^{36}\text{Cl}^-$ efflux, mean ± SEM, $n = 3$) and brought back to values similar to those observed in TNF-treated s-PMN.

Findings *a* and *b* confirm previously reported data (49). Findings *c* and *d* are new, suggesting that basal PMN adherence to FN and, more dramatically, TNF-stimulated adherence are sufficient conditions to trigger Cl^- movements in FN-PMN.

Interestingly, we found that two anti-TNF-R55 mAbs, i.e., mAb H398 and mAb htr-9, directly triggered a $^{36}\text{Cl}^-$ efflux from FN-PMN (60.5 and 59.5% after 20 min of incubation, respectively), comparable to that induced by TNF in the same cell population (69.5%). By contrast, an anti-TNF-R75 mAb, utr-1, was ineffective in this respect (33.9% $^{36}\text{Cl}^-$ efflux in the presence of mAb utr-1 vs 35.5% $^{36}\text{Cl}^-$ efflux of control). These findings are in line with previously reported data, showing that mAbs H398 and htr-9, but not mAb utr-1, mimic TNF-induced functional responses of FN-PMN, such as adherence and metabolic activation (34), and indicating that TNF-R55 signals also for Cl^- efflux.

Relationships between Cl^- Efflux, Adherence, and Activation of the Respiratory Burst in FN-PMN

It is well established that exposure of PMN to TNF mark-

edly enhances their adherence to immobilized FN, which is followed by spreading of the cells onto the surface and consequent activation of their oxidative metabolism (34, 38). We addressed the problem of the possible relationships between the TNF-induced Cl^- efflux, described in the previous paragraphs, and activation of PMN adherence and oxidative metabolism, by simultaneously running time course experiments of the three responses. The results are shown in Fig. 1. The time of the half-maximal response (MR_{50}) to TNF was 11 min for $^{36}\text{Cl}^-$ efflux (*a*), 20 min for adherence (*b*), and 28 min for O_2^- production (*c*), indicating that Cl^- efflux precedes adherence, and this, in turn, precedes the onset of the metabolic burst. TNF-induced PMN spreading, paralleling the metabolic response, began to be well detectable by 20 min of incubation (*d*). This finding confirmed previously reported data (39) and, in addition, indicated that spreading, like adherence and respiratory burst, is subsequent to the full activation of Cl^- efflux.

Chloride efflux preceded the onset of the other two responses even in unstimulated PMN. In fact, an appreciable release of $^{36}\text{Cl}^-$ was evident after 5 min of incubation, when adherence and O_2^- production were hardly detectable.

Effect of Cl^- Transport Inhibitors on TNF-induced Cl^- Efflux, Adherence, and Metabolic Activation of FN-PMN

The results reported in the previous paragraph raised the possibility of a causal relationship between Cl^- efflux and PMN adherence and metabolic activation on FN-coated surfaces. Such a possibility was tackled in experiments in which the effect on PMN adherence and activation of the respiratory burst of agents that may interfere with Cl^- efflux was investigated. Several drugs, belonging to diverse classes of Cl^- transport blockers, were tested: (*a*) EA and MA, both belonging to the phenoxyacetates family, have been reported to block chloride channels in rat and frog muscles (7); EA also inhibits ATP-dependent Cl^- transporters of human neutrophils (49, 51) and rat brain microsomes (26, 50); (*b*) bumetanide and furosemide, both members of the benzoates family; bumetanide is a blocker of Cl^- secretion in the epithelial cell lines T84 (11, 12) and MDCK (9), and furosemide acts on the same chloride transport mechanisms as EA (26, 49, 50, 51); (*c*) 9-AC, a polycyclic compound, known to inhibit Cl^- channels in canine tracheal epithelial cells (61), rat colonic enterocytes (13), and rat vascular smooth muscle cells (2); (*d*) DIDS, a member of the sulfonic acids family that blocks human erythrocyte anion channels (10) and chloride channels of mouse macrophages (43) and of the human histiocytic cell line U937 (28); (*e*) CHC, an inhibitor of the ATP-dependent and -independent chloride transport of human neutrophils (51) and of an ATP-independent Cl^- carrier of human erythrocytes (23).

After preliminary dose-response experiments, three of the above mentioned drugs, i.e., bumetanide, 9-AC, and DIDS, proved unsuitable for subsequent experiments since they affected per se one or more PMN responses even at concentrations that were ineffective or slightly effective on Cl^- efflux. In particular, 1 mM bumetanide sub-

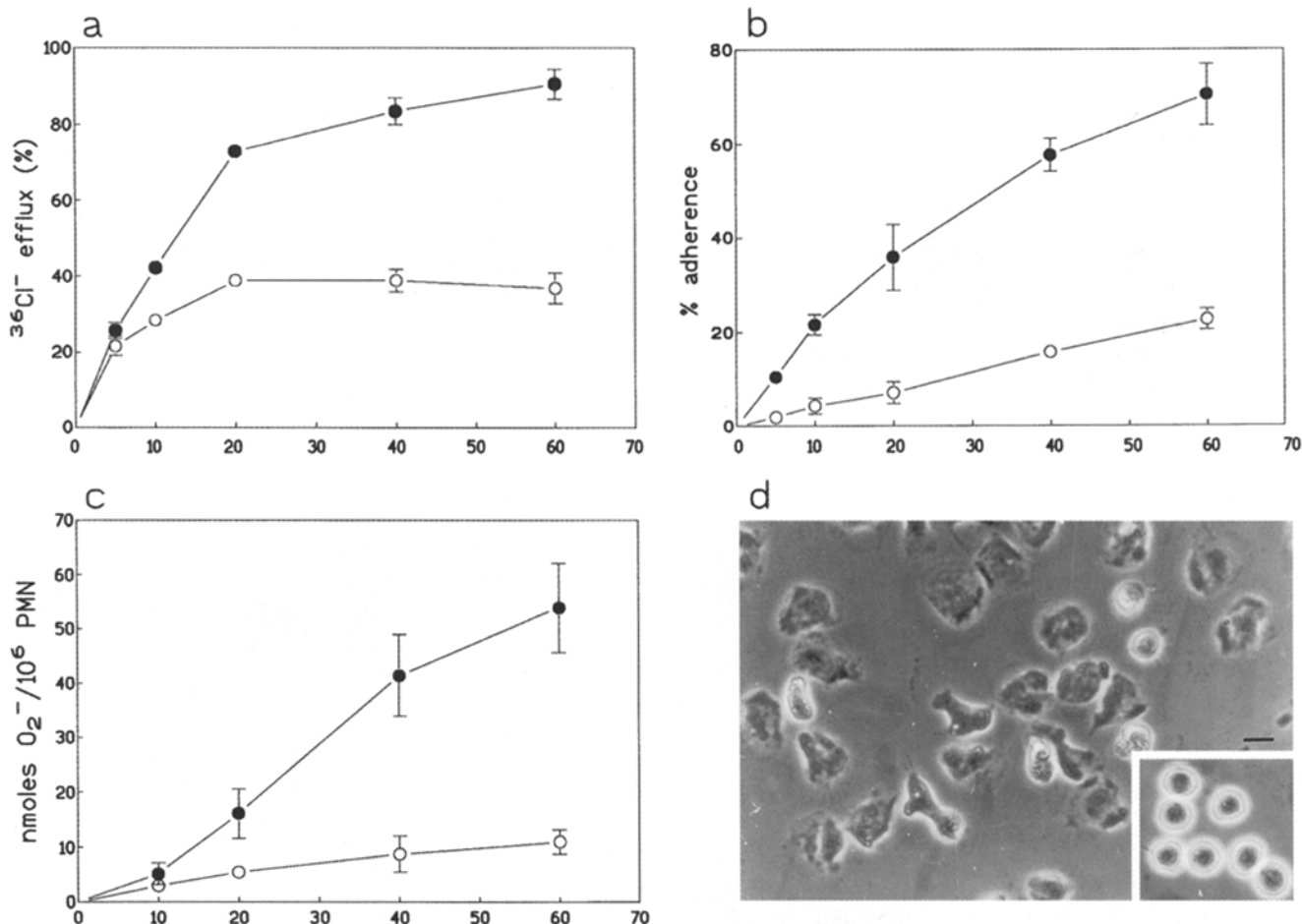


Figure 1. Kinetics of $^{36}\text{Cl}^-$ efflux, adherence, and O_2^- production by FN-PMN. $^{36}\text{Cl}^-$ efflux (a) was measured as described in the legend to Table I. Adherence (b) and O_2^- production (c) were assayed with aliquots of $^{36}\text{Cl}^-$ -loaded PMN used to measure $^{36}\text{Cl}^-$ efflux. Both the cell suspensions and the plates were prewarmed at 37°C for 5 min. For assay of PMN adherence, $50\ \mu\text{l}$ of PMN suspension ($1.5 \times 10^6/\text{ml}$) were added to FN-coated wells containing, in a $100\text{-}\mu\text{l}$ vol, $15\ \text{ng/ml}$ TNF (final concentration $10\ \text{ng/ml}$). On completion of the incubation, nonadherent PMN were removed by centrifuging the plates upside down, and the number of adherent cells was determined by assaying myeloperoxidase activity (14). The percentage of adherence was calculated using a calibration curve with known amounts of PMN. For assay of O_2^- production, $50\ \mu\text{l}$ of PMN suspension ($1.5 \times 10^6/\text{ml}$) was added to FN-coated wells containing, in a $100\text{-}\mu\text{l}$ vol, $0.18\ \text{mM}$ cytochrome c (final concentration $0.12\ \text{mM}$) and $15\ \text{ng/ml}$ TNF (final concentration $10\ \text{ng/ml}$). The plates were incubated at 37°C and read at the stated times in an automated microplate reader. Nanomoles of O_2^- were calculated as detailed in Materials and Methods. Data represent the means of four to seven experiments (in duplicate). (Bars) SEM; not shown when smaller than the symbols. (Open circles) Resting PMN; (closed circles) TNF-stimulated PMN. (d) TNF-induced PMN spreading onto FN-coated surfaces. (Inset) Unstimulated PMN. Bar, $5\ \mu\text{m}$.

stantially stimulated both resting (+ 95%) and TNF-induced (+ 40%) adherence, and had a minor enhancing effect (+ 15%) on TNF-induced O_2^- generation. Also, DIDS and 9-AC strongly stimulated (+ 50% to + 450%) resting and TNF-induced adherence and O_2^- production by FN-PMN at concentrations totally ineffective or only slightly inhibitory on Cl^- efflux (0.2 to $0.5\ \text{mM}$ for DIDS and 1 and $2\ \text{mM}$ for 9-AC, respectively).

As shown in Fig. 2, the remaining compounds tested were found to coordinately inhibit Cl^- efflux (upper panel), adherence, and O_2^- generation (lower panel) by TNF-stimulated FN-PMN. The IC_{50} were as follows: $2.5\ \text{mM}$ for CHC, $0.75\ \text{mM}$ for MA, and $0.085\ \text{mM}$ for EA. The IC_{50} of furosemide could not be exactly assessed, since the poor solubility of this compound hampered its use at concentrations $>2.5\ \text{mM}$. At this concentration, furo-

semide inhibited by $\sim 50\%$ all three responses. These results strongly indicate that, whatever the Cl^- transport mechanism involved, the efflux of this anion appears to play a major role in regulating adherence and O_2^- generation of TNF-stimulated FN-PMN.

Due to its potency on a molar basis (~ 10 times as high as MA, ~ 17 times as high as furosemide, and >25 times as high as CHC), EA was selected for subsequent studies. Fig. 3 shows that the effect of EA on TNF-induced $^{36}\text{Cl}^-$ efflux (upper panel), adherence (middle panel), and O_2^- production (lower panel) was dose dependent and that the pattern of inhibition was similar for the three responses.

Adhesion is the first of a series of sequential steps that lead to activation of the respiratory burst in TNF-treated PMN (32, 34, 37, 38). Impairment of cell adherence, in fact, is invariably accompanied by inhibition of the respiratory

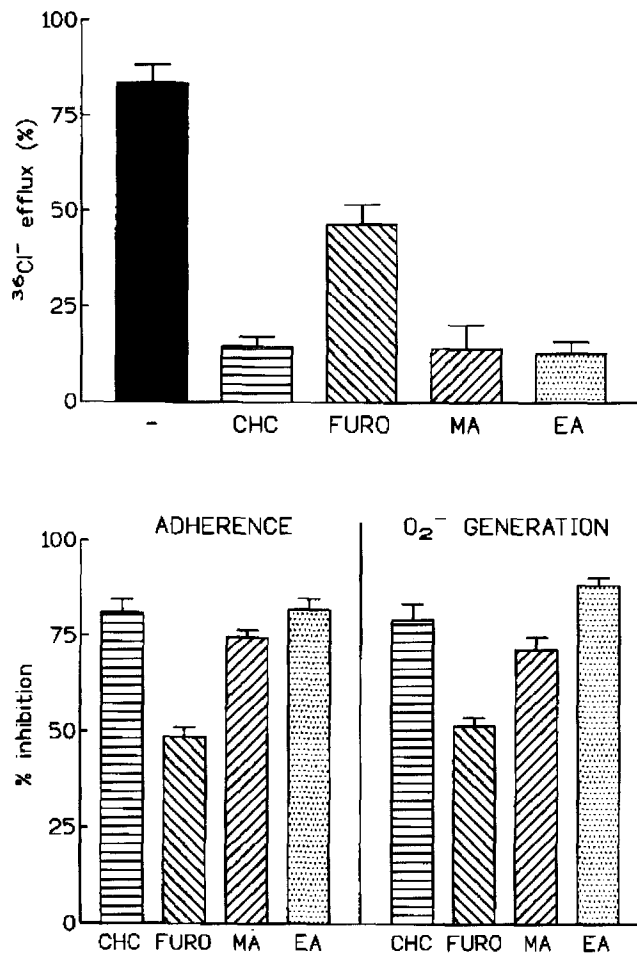


Figure 2. Effect of Cl^- transport inhibitors on TNF-induced $^{36}\text{Cl}^-$ efflux, adherence, and O_2^- production of FN-PMN. Assays of $^{36}\text{Cl}^-$ efflux (upper panel), adherence, and O_2^- production (lower panels) were as described in detail in the legends to Table I and Fig. 1, respectively. PMN were preincubated in suspension with control buffer or the drugs (CHC: 5 mM; furosemide: 2.5 mM; MA: 1.5 mM; EA: 0.150 mM) for 10 min at 37°C in a shaking water bath, and then added to FN-coated wells. Data represent the means of three to seven experiments (in duplicate). (Bars) SEM.

burst (32, 34, 37, 38). The results of Fig. 2 and 3, showing that EA pretreatment of PMN inhibited both adherence and O_2^- generation, agree with this notion and indicate that Cl^- efflux is important for the TNF-induced adherence of PMN to FN.

It has been recently shown that adherence, though necessary, is not sufficient for PMN to respond with a respiratory burst when exposed to TNF, and that a postadhesive event, i.e., spreading, is also required (34, 37, 38). Since in our model, adhesion to FN was found not only to enhance but also to prolong TNF-induced Cl^- efflux (see Table I and Fig. 1), we wondered whether Cl^- movements, apart from being involved in the initial adhesion step, could be relevant also in the subsequent spreading and metabolic activation.

To test for this possibility, we first incubated PMN in suspension with TNF, to allow Cl^- efflux and $\beta 2$ integrin activation to occur. Subsequently, the cells were plated in

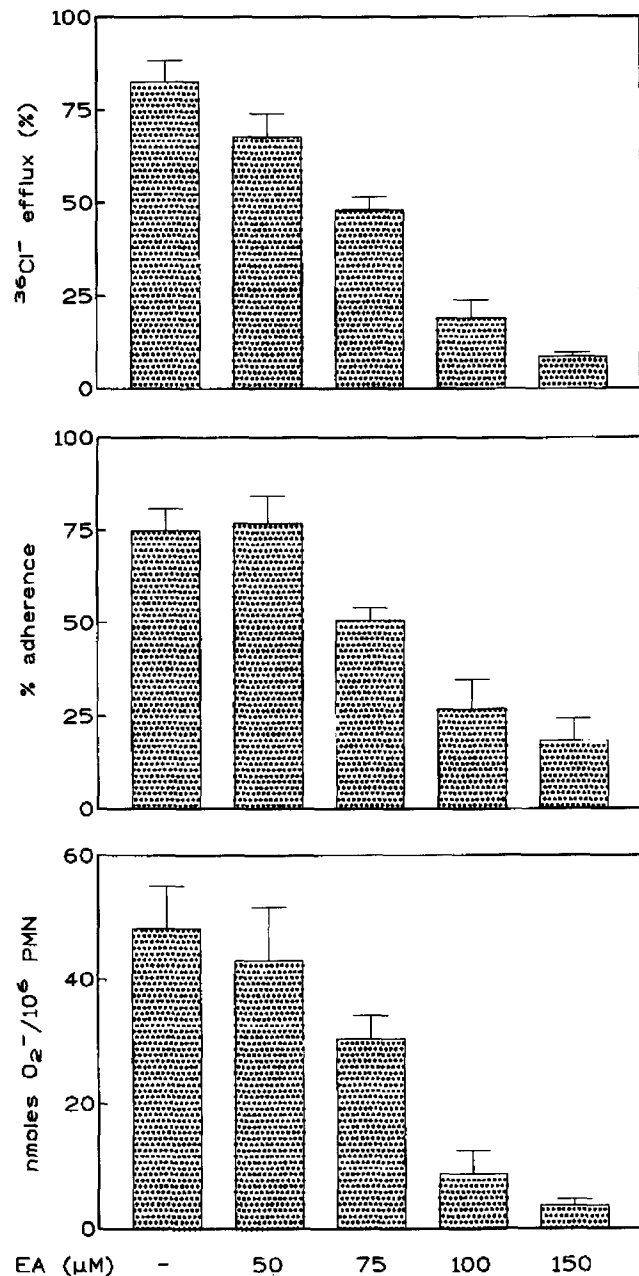


Figure 3. Effect of different concentrations of EA on $^{36}\text{Cl}^-$ efflux (upper panel), adherence (middle panel), and O_2^- generation (lower panel) of TNF-stimulated FN-PMN. Assays of $^{36}\text{Cl}^-$ efflux, adherence, and O_2^- production were as described in the legends to Table I and Fig. 1. PMN were preincubated with control buffer or different concentrations of EA for 10 min at 37°C in a shaking water bath, and then placed into FN-coated wells in the presence of 10 ng/ml TNF. Data represent the means of three to eight experiments (in duplicate). (Bars) SEM.

EA-containing wells, to prevent any further Cl^- release, and adherence and O_2^- production were assayed.

The results of these experiments are shown in Table II and Fig. 4. PMN, exposed in suspension to TNF and then plated in EA-containing wells, adhered to FN to the same extent as did TNF-stimulated PMN plated in wells in the absence of EA ($59.1 \pm 11.1\%$ adherence in the presence

Table II. Effect of EA on Adherence and O₂⁻ Production of FN-PMN

Pretreatment in suspension	Treatment in wells	Adherence	O ₂ ⁻ /10 ⁶ PMN
		%	nmol
None	TNF	64.7 ± 5.2	40.7 ± 6.1
EA 100 μM	TNF	25.2 ± 8.0	4.3 ± 2.2
TNF	None	66.0 ± 8.7	38.6 ± 7.1
TNF	EA 100 μM	59.1 ± 11.1	20.1 ± 5.7*

PMN were pretreated in suspension at 37°C for 10 min with control buffer or 100 μM EA, and for 15 min with 10 ng/ml TNF. The cells were then seeded in FN-coated wells, and adherence and O₂⁻ production were measured after a 60-min incubation as described in the legend to Fig. 1. For other details, see Materials and Methods. Data represent the means ± SEM of five experiments (in duplicate).

*The difference between TNF-induced O₂⁻ production by PMN treated or not in the wells with EA was statistically significant ($P < 0.015$), as calculated by one-tailed *t* test on paired data.

of EA vs 66.0 ± 8.7% adherence in the absence of EA, mean ± SEM, $n = 5$), but their respiratory burst was markedly lower (20.1 ± 5.7 vs 38.6 ± 7.1 nmol of O₂⁻, mean ± SEM, $n = 5$; $P < 0.015$ by one-tailed *t* test on paired data). Scanning EM observations revealed that cells treated in suspension with TNF and then plated in EA-containing wells (Fig. 4 *b*) spread onto FN much less than did cells treated in suspension with TNF and plated without EA (Fig. 4 *a*). A quantitative evaluation of the inhibitory effect of EA on TNF-induced PMN spreading on FN-coated surfaces was carried out by phase-contrast microscopy examination of cells treated with different concentrations of the compound. The results, shown in Table III, clearly demonstrated that EA inhibited PMN spreading in a dose-dependent fashion. Taken altogether, these results suggest that Cl⁻ efflux, besides adherence, may regulate both spreading and metabolic activation of TNF-treated FN-PMN and further support the current view that spreading, rather than a mere adhesion, is essential for the metabolic activation of these cells (18, 37, 41).

A possible toxic effect of EA on PMN was ruled out on the basis of experiments, not reported here in detail, demonstrating that (*a*) PMN treated with 50 to 250 μM EA were >95% viable, as judged by the Trypan blue exclusion test; (*b*) the inhibitory effect of EA was reversible, since PMN treated for 10 min with 100 or 150 μM EA, washed twice and tested for adherence and O₂⁻ production on FN, responded to TNF to the same extent as did untreated PMN; (*c*) s-PMN pretreated with 100 or 150 μM EA responded to either the chemotactic peptide FMLP (10⁻⁷ M)

Table III. Effect of Different Concentrations of EA on TNF-induced Spreading of PMN on FN-coated Surfaces

[EA] μM	Spread PMN	Not spread PMN
	%	%
—	79.6 ± 5.2	20.4 ± 2.1
50	63.9 ± 3.3	36.1 ± 3.3
75	39.4 ± 3.0	60.6 ± 4.1
100	18.6 ± 1.5	81.4 ± 3.8
150	10.6 ± 0.8	89.4 ± 2.4

Assay conditions were as described in the legend to Fig. 3. For other experimental details, see Materials and Methods. Data are the means ± SEM of three to five determinations carried out by examining a corresponding number of randomly chosen microscopic fields. At least 300 cells per experimental condition were scored.

or PMA (20 ng/ml) with a respiratory burst strictly comparable to that of untreated PMN (30.1 and 29.0 nmol of O₂⁻ of FMLP-stimulated PMN treated with 100 and 150 μM EA, respectively, vs 26.8 nmol of O₂⁻ of untreated cells; 180.1 and 176.9 nmol of O₂⁻ of PMA-stimulated PMN treated with 100 and 150 μM EA, respectively, vs 166.9 nmol of O₂⁻ of untreated cells).

Unstimulated PMN Suspended in Cl⁻-free Medium Release Chloride Ions, Adhere to FN, and Have an Activated Respiratory Burst

The results of the previous paragraphs suggest that Cl⁻ efflux precedes and is related to increased adherence, spreading, and metabolic burst of TNF-stimulated FN-PMN. If correct, the expectation is that Cl⁻ efflux induced by means other than TNF treatment would mimic those three responses to TNF.

This was done by suspending the PMN in a Cl⁻-free medium, i.e., a medium where Cl⁻ was replaced by the cell-impermeant anion glucuronate (G-buffer). In such a medium, Cl_i⁻ was shown to progressively decrease as a consequence of a forced chloride efflux down its concentration gradient (52).

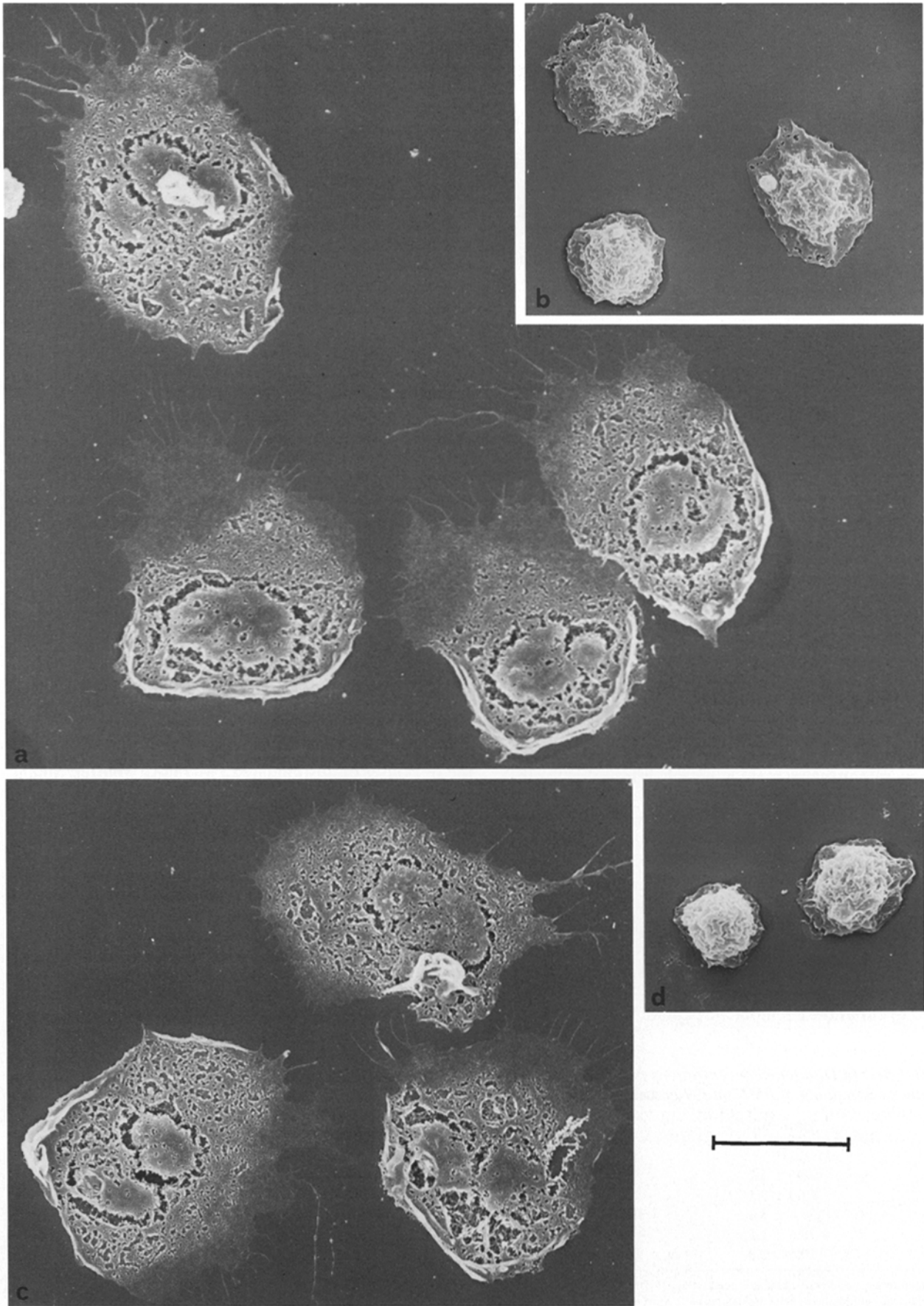
Fig. 5 shows that FN-PMN in G-buffer exhibited a massive efflux of ³⁶Cl⁻ accompanied by a decrease of ³⁶Cl_i⁻ (*d*), a considerable increase in adherence, and a vigorous respiratory burst (*e* and *f*, respectively). All three responses were strictly comparable to those observed in TNF-treated PMN in Cl⁻ buffer (*a*, *b*, and *c*, respectively). Similar results were obtained when Cl⁻ was replaced by other cell-impermeant anions, e.g., gluconate, known to cause progressive efflux of Cl⁻ from amphibian neurones (1), or glutamate. In particular, PMN suspended in glutamate-containing buffer increased their adherence and O₂⁻ production by 97 and 231%, respectively, over the responses of unstimulated PMN in Cl⁻-buffer. Similar, although less pronounced, was the enhancement of these PMN responses in gluconate-containing buffer (+70% for adherence and +163% for O₂⁻ production).

Fig. 6 shows that the addition of EA to FN-PMN suspended in G-buffer caused a considerable inhibition of ³⁶Cl⁻ efflux (*left panel*), adherence (*middle panel*), and O₂⁻ production (*right panel*), further supporting the hypothesis that efflux of Cl⁻ is related to the activation of the adhesive and metabolic response of FN-PMN.

Fig. 4 *c* is a scanning EM micrograph showing that unstimulated FN-PMN in G-buffer also spread in a fashion similar to that of TNF-stimulated PMN in Cl⁻ buffer (Fig. 4 *a*). The extent of spreading may be more easily appreciated if compared to the roundish appearance of unstimulated PMN in Cl⁻ buffer (Fig. 4 *d*). Spreading of EA-treated FN-PMN in G-buffer was also markedly reduced (not shown).

Discussion

The present study is the first one that analyzes chloride ion movements in human PMN residing on biologic surfaces and demonstrates a relationship between modifications of the intracellular chloride levels and PMN responses triggered by TNF, such as adherence, spreading, and activation of the respiratory burst.



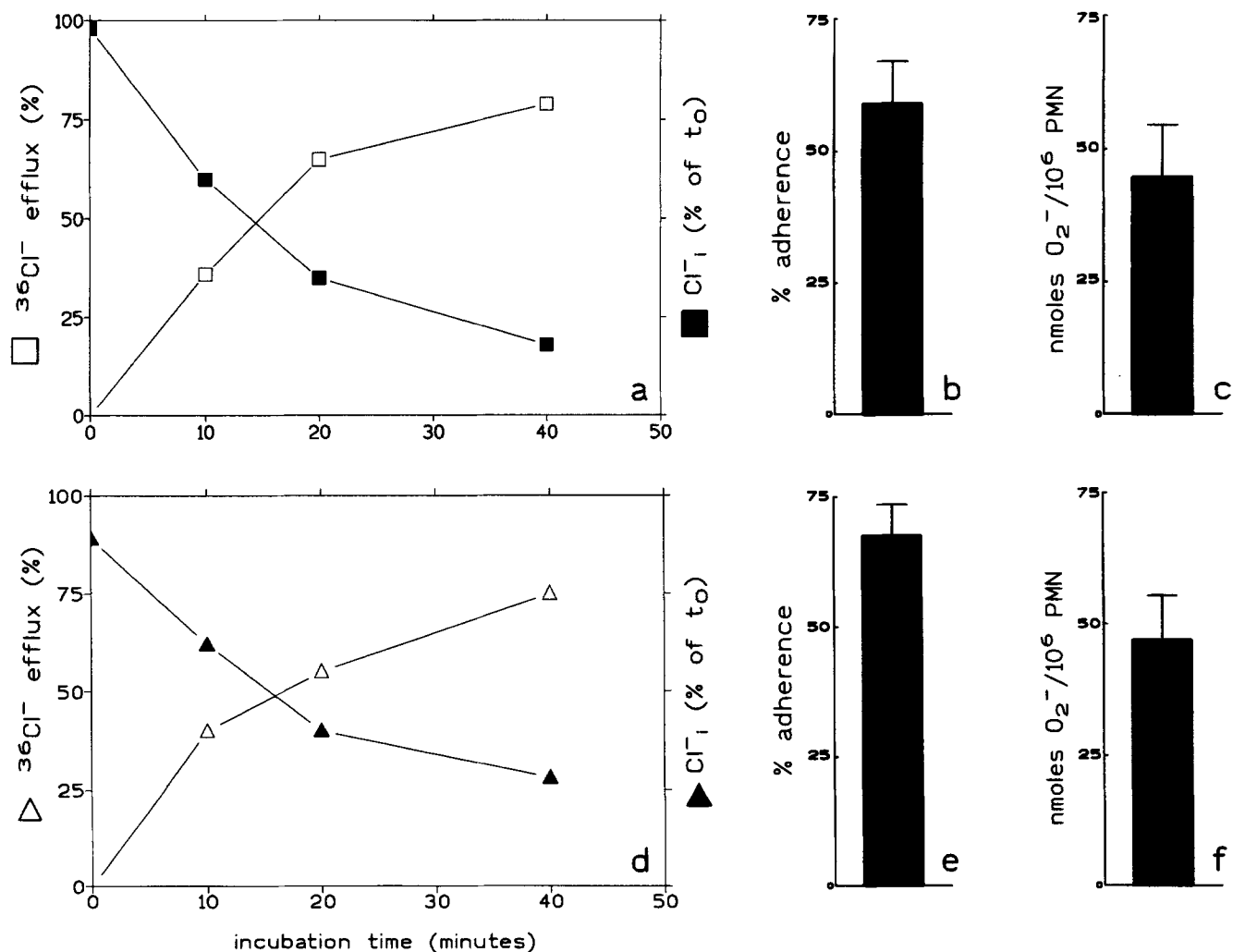


Figure 5. Effect of Cl^- -free buffer on $^{36}\text{Cl}^-$ efflux, $^{36}\text{Cl}^-_i$, adherence, and O_2^- generation of FN-PMN. $^{36}\text{Cl}^-$ efflux, $^{36}\text{Cl}^-_i$, adherence, and O_2^- generation were assayed in TNF-stimulated FN-PMN bathed in Cl^- -containing buffer (a, b, and c, respectively; TNF final concentration 10 ng/ml) and in unstimulated FN-PMN bathed in Cl^- -free glucuronate-containing buffer (d, e, and f, respectively). Assays of $^{36}\text{Cl}^-$ efflux and $^{36}\text{Cl}^-_i$ were as described in the legend to Table I. Adherence and O_2^- generation were measured after a 60-min incubation at 37°C as detailed in the legend to Fig. 1. Each point in the graphs shown in a and d represents the mean of duplicate assays of one experiment representative of three that gave similar results. (a) $^{36}\text{Cl}^-$ efflux, (open squares); $^{36}\text{Cl}^-_i$, (closed squares); (d) $^{36}\text{Cl}^-$ efflux, (open triangles); $^{36}\text{Cl}^-_i$, (closed triangles). In b, c, e, and f, the data represent the means \pm SEM of five experiments (in duplicate).

It has been recently shown that Cl^- efflux is a phenomenon that occurs early in PMN treated in suspension with a variety of priming and/or activating agonists (49). TNF, along with granulocyte/macrophage-colony stimulating factor, was identified as the most potent stimulator of Cl^- efflux, suggesting that chloride release may represent an important event in the signal transduction pathway activated by this cytokine. Nevertheless, the role of Cl^- movements in the modulation of PMN responses to TNF is not defined yet.

The model of the PMN residing on surfaces coated with FN permitted us to show the following:

(a) Basal adhesion of PMN to FN stimulates per se a net Cl^- efflux, leading to a decrease of Cl^-_i . Such an efflux was likely mediated by the $\beta 2$ -independent basal adhesion of PMN to FN, since it was only minimally affected by the anti- $\beta 2$ mAb 60.3.

(b) TNF greatly amplifies and prolongs Cl^- efflux from FN-PMN. Much of this efflux relies on $\beta 2$ -dependent adhesion, since mAb 60.3, which strongly decreases $\beta 2$ -dependent adherence of TNF-treated PMN to FN (this paper; 34, 59), brings the efflux back to about the values of PMN stimulated by TNF in suspension. This suggests that engagement of the $\beta 2$ integrins (previously activated by

Figure 4. Scanning EM micrographs of PMN residing on FN-coated surfaces. PMN in Cl^- -containing buffer were preincubated in suspension for 15 min at 37°C with 10 ng/ml TNF (a and b) and transferred onto FN-coated surfaces in the absence (a) or in the presence (b) of 100 μM EA. (c) Appearance of unstimulated PMN bathed in Cl^- -free glucuronate-containing buffer. (d) Appearance of unstimulated PMN bathed in Cl^- -containing buffer. Bar, 10 μm .

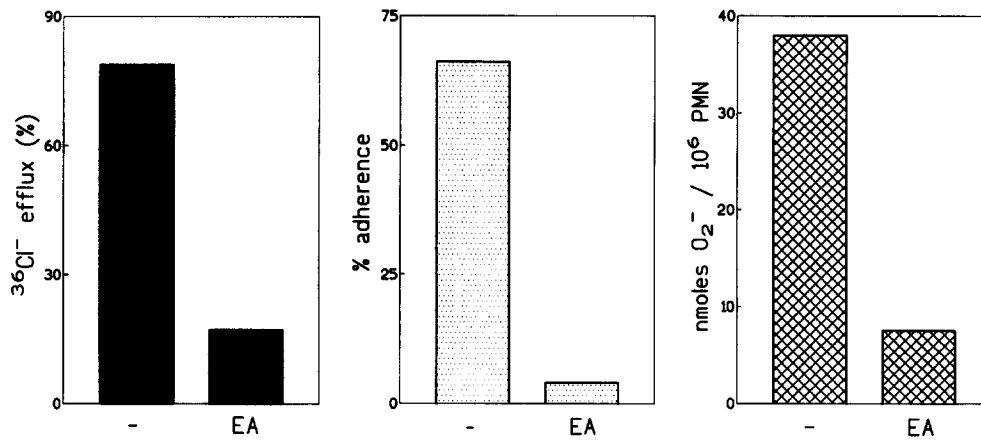


Figure 6. Effect of EA on $^{36}\text{Cl}^-$ efflux (*left*), adherence (*middle*), and O_2^- generation (*right*) of unstimulated FN-PMN bathed in Cl^- -free gluconate-containing buffer. The cells were preincubated in suspension for 10 min at 37°C with control buffer or $200\ \mu\text{M}$ EA, transferred to FN-coated wells, and then incubated for an additional 60 min. $^{36}\text{Cl}^-$ efflux, adherence, and O_2^- generation were measured thereafter, as detailed in the legends to Table I and Fig. 1. Data are representative of three independent experiments (in duplicate).

TNF) by their counter-receptor on FN signals per se for an additional Cl^- efflux.

(c) Cl^- efflux induced by means other than TNF treatment, i.e., by placing FN-PMN in Cl^- -free media, was followed by a qualitative and quantitative mimicry of TNF effects, i.e., increased adherence, spreading, and activation of the metabolic burst. These findings provide new evidence in favor of a close correlation between lowering of Cl_i^- and activation of PMN functions and are in agreement with previous results by Grinstein and co-workers (22), showing that suspension of permeabilized PMN in Cl^- -depleted media elicited protein phosphorylation, actin polymerization, secretion of lysozyme, and a respiratory burst, and those by Fittschen and Henson (17), showing that PMN bathed in a Cl^- -free medium selectively and consistently released specific granules.

(d) Inhibition of the Cl^- efflux from either TNF-treated FN-PMN or FN-PMN in Cl^- -free media by the Cl^- transport inhibitor EA resulted in almost complete inhibition of adherence, spreading, and metabolic burst.

(e) The use of EA as an inhibitor of Cl^- efflux permitted us also to establish that the release of Cl^- may be independently linked to adhesion on one side, and spreading and activation of the metabolic burst on the other. In fact, if PMN are incubated in suspension with TNF (in this step, Cl^- efflux and activation of the integrins have occurred) and then placed to adhere to FN in the presence of EA, cell spreading and the metabolic burst are considerably impaired, while adhesion is not (Table II), since it relies on the integrins activated in suspension. This indicates that the Cl^- efflux triggered by the integrin engagement in the adhesion step has a role in the two subsequent events, i.e., spreading and activation of the burst.

We believe that the results discussed above can be incorporated in the following coherent picture. When TNF contacts FN-PMN, the first change that occurs is a Cl^- efflux that can be clearly detected as early as 5 min after TNF addition. This is followed by adherence but not necessarily by spreading and activation of the burst. Then, adhesion signals for an additional Cl^- efflux that makes the cells proceed toward spreading and metabolic activation.

How does TNF trigger Cl^- extrusion? Kagan et al. have suggested that TNF itself in the trimeric form may act as an ion channel within the plasma membrane of histiocytic lymphoma cells (27). This does not seem to apply to our situation, since the effects of TNF treatment could be fully reproduced with two agonistic anti-TNF-R55 mAbs, indicating that the phenomena we have investigated are receptor mediated. Further studies are needed to elucidate the connections between the postreceptor events and the stimulation of Cl^- release.

Chloride movements in PMN may occur through diverse pathways. Detailed studies by Simchowicz and co-workers have identified at least three different mechanisms regulating Cl^- traffic in resting PMN, i.e., passive electrodiffusion, a Cl^-/Cl^- exchanger, and an ATP-driven Cl^- transport system that is responsible for the unusually high intracellular Cl^- concentration (51). Stretch-activated Cl^- channels have been demonstrated to operate in resting PMN subjected to hypotonic stress (54). More recently, voltage-gated and Ca^{2+} -activated Cl^- channels, stimulated by FMLP and PMA (47) or TNF (48), respectively, have been identified by the patch-clamp technique in PMN. Also, PMN have been shown to belong to a category of nonepithelial cells in which low levels of mRNA transcripts of the cystic fibrosis transmembrane regulator gene have been detected (63), but the formal proof that this Cl^- channel is expressed on the PMN is lacking so far.

Which one(s) of these pathways is (are) involved in the TNF-mediated and β_2 integrin-mediated Cl^- extrusion? This is under investigation, as is the nature of the ion(s) cotransported or transported in parallel with Cl^- . At this stage of the work, we can only exclude any role for the cystic fibrosis transmembrane regulator, since PMN isolated from two cystic fibrosis patients responded normally to TNF (unpublished observations).

Another question concerns the link between decrease in Cl_i^- and activation of PMN adhesiveness, spreading, and respiratory burst. Since cytoplasmic pH (25, 53), membrane potential (30, 55, 56), and cell volume (24, 25, 54, 60, 62) have been shown to be controlled by Cl^- movements in diverse cell types, experiments are in progress to pick up

possible connections between one or more of such parameters and activation of proadhesive molecules, reorganization of the cytoskeleton network, and assembly of the O_2^- -forming NADPH oxidase, which are the basic changes underlying activation of the PMN functions studied in this paper.

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