Tumor Necrosis Factor and CD11/CD18 (β2) Integrins Act Synergistically to Lower cAMP in Human Neutrophils

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Abstract. The ability of neutrophils (PMN) to undergo a prolonged respiratory burst in response to cytokines such as tumor necrosis factor-α (TNF) depends on expression of CD11/CD18 (β2) integrins and interaction with matrix protein-coated surfaces (Nathan, C., S. Srimal, C. Farber, E. Sanchez, L. Kabbash, A. Asch, J. Gailit, and S. D. Wright. 1989. *J. Cell Biol.* 109:1341–1349). We tested the hypothesis that changes in cAMP mediate the joint action of cytokines and integrins. When plated on FBS- or fibrinogen-coated surfaces, PMN responded to TNF with a sustained fall in intracellular cAMP. This did not occur without TNF; in suspended PMN; in PMN treated with anti-CD18 mAb; or in PMN genetically deficient in β2 integrins. A preceding fall in cAMP appeared essential for TNF to induce a respiratory burst, because drugs that elevate cAMP blocked the burst if added any time before, but not after, its onset. Adenosine analogues and cytochalasins also block the TNF-induced respiratory burst if added before, but not after, its onset. Both also blocked the TNF-induced fall in cAMP.

The effect of cytochalasins led us to examine the relationship between cAMP and actin reorganization. The same conditions that led to a sustained fall in cAMP led at the same time to cell spreading and the assembly of actin filaments. As with the respiratory burst, cAMP-elevating agents inhibited TNF-induced cell spreading and actin filament assembly if added before, but not after, spreading began.

Thus, occupation of TNF receptors and engagement of CD18 integrins interact synergistically in PMN to promote a fall in cAMP. The fall in cAMP is closely related to cell spreading and actin reorganization. These changes are necessary for TNF to induce a prolonged respiratory burst. We conclude that integrins can act jointly with cytokines to affect cell shape and function through alterations in the level of a second messenger, cAMP.

NEUTROPHILS (PMN)[1] adherent to extracellular matrix proteins react to peptide agonists differently than PMN in suspension (25, 34–36, 38, 45). For example, the respiratory burst of adherent PMN in response to tumor necrosis factor-α (TNF) (16, 29, 34, 36, 45), other cytokines (16, 34, 35), N-formylated peptides (16, 34), or complement component C5a (Nathan, C., manuscript in preparation) is delayed in onset by 15–90 min, whereas suspended PMN respond to soluble stimuli in <1 min. Thereafter, adherent PMN sustain the respiratory burst 10–100 times longer and produce 10–100 times more O_2 intermediates than PMN in suspension (34, 35).

Little is known about the morphologic or molecular counterparts of the adhesion dependence of PMN responses. The cytokine-induced PMN respiratory burst requires CD11/CD18 (β2) integrins (36). These serve as receptors for some matrix proteins and/or as mediators of responses to the ligation of others (36). The onset of the TNF-induced respiratory burst appears to depend on the ability of actin to polymerize, in that cytochalasins abort the response if added during but not after the lag period (34). Likewise, adenosine and nonmetabolizable adenosine analogs inhibit the peptide-induced respiratory burst, but only if added before its onset (16). The mechanisms whereby cytochalasins and adenosine analogues inhibit PMN responses are unknown.

Pharmacologic elevation of cAMP can block PMN chemotaxis, degranulation, and superoxide release (3, 5, 10, 32, 37, 39, 60, 62), inhibit the ability of the T cell antigen receptor to induce an increase in the avidity of the CD11a/CD18 (β2) integrin for its ligand, ICAM-1 (18), and promote collapse of microfilaments in fibroblasts, leading to rounding of the cells (27, 53). These observations prompted us to investigate whether adherent, TNF-treated PMN might sustain a fall in intracellular cAMP in association with cell spreading, assembly of actin filaments, and secretion of reactive oxygen intermediates.

1. *Abbreviations used in this paper*: CSF-GM, colony-stimulating factor for granulocytes/macrophages; DHCB, dihydrocytochalasin B; IBMX, isobutylmethylxanthine; KRPG, Krebs-Ringer phosphate buffer with glucose; PMN, neutrophilic polymorphonuclear leukocyte(s); TNF, tumor necrosis factor-α .

Materials and Methods

Preparation of PMN and Measurement of H_2O_2 Release

These methods were recently described (36). In brief, PMN were isolated from normal adult donors' heparinized venous blood on a one-step, modified Ficoll-Hypaque gradient without exposure to hemolytic conditions and resuspended in ice-cold Krebs-Ringer phosphate buffer with glucose (KRPG). Where indicated, PMN were isolated from a 13-mo-old girl with leukocyte adhesion deficiency, the diagnosis of which was documented as described in reference 36. The suspensions contained 96.71 ± 0.36% neutrophils, 2.96 ± 0.38% eosinophils, 0.24 ± 0.10% lymphocytes, and 0.08 ± 0.06% monocytes (mean ± SEM for 16 experiments). Where indicated, pretreatment with mAb (30 μg/ml) lasted 70–90 min at 37°C while the PMN were agitated at 150 horizontal cycles per min with or without 8 end-over-end rotations per min in polypropylene microfuge tubes precoated with FBS (Hyclone Systems, Logan, UT). To measure H_2O_2 release, 1.5×10^4 PMN in 20 μl were plated per 6-mm-diam well in FBS- or fibrinogen-precoated Primaria or conventional polystyrene 96-well plates (Falcon Labware, Becton-Dickinson & Co., Oxnard, CA). The prewarmed microplate wells contained 100 μl KRPG with 4 nmol scopoletin, 0.44 purpurogallin units/ml horseradish peroxidase, and 1 mM NaN_3 to inhibit myeloperoxidase (reaction mixture) plus agonists in 10 μl KRPG or KRPG alone (final volume, 130–140 μl). The plates were incubated for up to 2 h at 37°C in air. Horseradish peroxidase–catalyzed oxidation of scopoletin was recorded at 15-min intervals in a plate-reading fluorimeter.

Intracellular cAMP

Flat-bottomed polystyrene trays with 16-mm-diam wells (no. 3047; Falcon Labware) were left uncoated, or precoated with 200 μl FBS or 500–800 μg of fibrinogen (Sigma Chemical Co., St. Louis, MO) in 200 μl RPMI 1640 (Kansas City Biologicals, Lenexa, KS) for 1–2 h at 37°C in 5% CO_2, then flicked dry and washed three times with 0.9% NaCl. Reaction mixture (see above) was added (200 μl), followed by test agents in KRPG or KRPG alone (totaling 40 μl), and the plates were prewarmed for 30 min at 37°C in air. PMN ($7–9 \times 10^5$ in 30 μl) were added to triplicate wells and the plates incubated at 37°C in air. Alternatively, reagents and cell suspensions in the same volumes as in plates were incubated in triplicate in FBS-precoated polypropylene microfuge tubes under rotation as above. At designated times, wells or tubes received 500 μl absolute ethanol precooled to −20°C (final concentration, 65%) (19). Time 0 samples were prepared by adding PMN to ethanol-containing incubates in vessels precooled to −20°C. Samples were held at −20°C for 1 h, then microcentrifuged (8,000 *g*, 5 min, room temperature). The supernatants were heated and evaporated under vacuum (Speed-Vac; Savant Instrument Co., Farmingdale, NY) and the lyophilates stored at −20°C until acetylation and RIA according to the manufacturer's protocol (Dupont NEN Research Products, Boston, MA). Cell-free samples were included in each experiment. Their values (averaging 9.8 ± 1.7% of the time 0 values) were subtracted.

Assessment of Cell Spreading and Adherence

PMN (2×10^5 in 30 μl) were added to 16-mm-diam wells prepared as above. The plates were incubated at 37°C in air for various times before the cells were fixed by addition of an equal volume of 2.5% glutaraldehyde in KRPG at 37°C. To score the proportion of adherent cells, the monolayers were examined with an inverted microscope without further manipulation. For photomicroscopy of adherent cells, the wells contained FBS-coated glass coverslips. After the monolayers were fixed, the coverslips were dipped in warm KRPG and inverted into a polyvinyl alcohol/glycerol mounting medium (40) on glass slides (the time course and extent of TNF-induced spreading and H_2O_2 release on FBS-coated glass coverslips closely matches that on FBS-coated plastic). To stain actin, monolayers were fixed and permeabilized with 3.7% formaldehyde and 0.01% lysophosphatidylcholine (Sigma Chemical Co., St. Louis, MO) and incubated for 20 min at 4°C in the dark with rhodamine-phallacidin (Molecular Probes, Junction City, OR). To stain tubulin, monolayers were fixed and permeabilized in methanol for 5 min at −20°C, washed three times with PBS-1% FBS, incubated for 1 h at 37°C with a 1:500 dilution of hybridoma culture supernatant of mouse anti-α-tubulin mAb (ICN ImmunoBiologicals, Lisle, IN), washed three times as before, incubated 1 h at 37°C with a 1:100 dilution of rhodamine-conjugated goat IgG anti-mouse IgG and IgM (Jackson Immunoresearch Laboratories, West Grove, PA), and washed twice in buffer and twice in distilled water. To estimate the ability of PMN to resist forceful dislodgment, 10^5 PMN were plated in 6-mm-diam FBS-coated wells as for the H_2O_2 assay. After 30 or 60 min at 37°C, the wells were flicked dry and flooded with 0.9% NaCl three times. Each well then received 100 μl reaction mixture for measurement of adherent cell protein as described (15).

Other Reagents

Dibutyryl cAMP, dibutyryl cGMP, isobutylmethylxanthine (IBMX), forskolin, dihydrocytochalasin B (DHCB), and 2-chloroadenosine were from Sigma Chemical Co.

Results

Joint Control of cAMP by TNF and Contact with a Protein-coated Surface

The level of cAMP in ice-cold PMN in suspension (0.52 ± 0.05 pmol/10^6 cells) matched values reported for untreated PMN in other investigations (reviewed in reference 32). In five experiments, the average value of cAMP in PMN suspended at 37°C for 45–60 min was no different than at time 0, whether or not the cells were exposed to TNF (Table I).

Results were strikingly different when PMN were added to stationary wells having flat bottoms coated with FBS. Fig. 1 compares the time course of the cAMP response with that of the respiratory burst in the same experiment. Over the first 2 min of contact with FBS-coated plastic, intracellular cAMP fell to <0.1 pmol/10^6 cells, whether or not TNF was present. Thereafter, untreated PMN gradually restored their cAMP to approximately the same level as PMN in suspension, and maintained this level through 2 h. However, in TNF-treated PMN, the recovery of cAMP ceased at ~10 min. At that point, cAMP began to drift downward again. By 45 min, as summarized in Table I, cAMP in TNF-treated, adherent PMN averaged 46 ± 4.8% (mean ± SEM, five experiments) lower than in suspended PMN. As the respiratory burst ended, cAMP in TNF-treated, adherent PMN returned to or exceeded the level in the controls. For simplicity, the difference in cAMP levels between TNF-treated and untreated PMN in the interval from ~10–90 min will be referred to as a "fall" in cAMP, although it could also be regarded as a TNF-induced delay in the recovery of cAMP from its early decline.

The concentration of TNF causing a half-maximal fall in cAMP at 45 min in adherent PMN ranged from 0.3–0.5 ng/ml, similar to the concentration causing half-maximal H_2O_2 release measured simultaneously (Fig. 2).

*Table I. Combined Effects of TNF and Adherence to FBS-coated Plastic on cAMP Levels in PMN**

Status	TNF	cAMP
	ng/ml	*pmol/10^6 PMN*
Suspended	0	0.507 ± 0.042‡
Suspended	100	0.507 ± 0.058
Adherent	0	0.446 ± 0.047
Adherent	100	0.272 ± 0.034

* Results are for 45-min incubations at 37°C, except for two experiments with suspended PMN at 60 min. All plates (adherent cells) and tubes (suspended cells) were precoated with FBS.
‡ Means ± SEM of mean results from five experiments, each in triplicate. Time zero values averaged 0.518 ± 0.049 pmol cAMP/10^6 PMN.

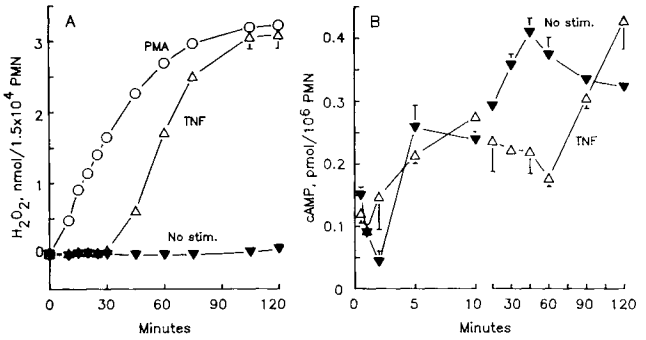

Figure 1. Time course of changes in cAMP levels in adherent PMN in comparison with the respiratory burst. Both assays were performed simultaneously with the same PMN preparation in separate plates made from the same kind of polystyrene and precoated with FBS. (*A*) Respiratory burst of PMN left untreated (no stim.; *inverted triangles*) or stimulated with TNF (100 ng/ml; *upright triangles*) or PMA (100 ng/ml; *circles*). The lag period in H_2O_2 secretion in response to TNF was 37 min, as estimated by back-extrapolation of the maximal rate to the baseline. Results are means ± SEM of triplicates; where error bars are not seen, they fall within the symbols. (*B*) cAMP levels in PMN cultured alone (no stim.; *inverted triangles*) or with TNF (100 ng/ml; *upright triangles*). Note that the abscissa is divided into two time scales. Results are means ± SEM of triplicates; where error bars are not seen, they fall within the symbols. Time 0 values were not successfully measured in this experiment, because cold EtOH was added to prewarmed reaction plates, followed by cells. It was subsequently determined in controlled experiments that this method was not adequate to prevent rapid changes in cAMP. True time 0 values (0.52 ± 0.05 pmol/10^6 PMN) were determined in five subsequent experiments by chilling plates containing the mixture of EtOH and reaction buffer to −20°C before adding PMN. The experiment shown was repeated with nearly identical results.

The TNF-induced Fall in cAMP Depends on CD11/CD18 (β2) Integrins

The above results were for PMN on FBS-precoated wells. In other experiments, wells were coated with fibrinogen, for which CD11/CD18 molecules serve as the only known receptors on PMN (56). In this setting, the changes in cAMP followed the same kinetics as in Fig. 1*B* (not shown), such that cAMP fell 34–35% at 45 min in TNF-treated compared with control PMN (Table II).

In earlier work (36), we tested 16 antibodies for their ability to inhibit the TNF-induced respiratory burst of PMN, including reagents directed against integrins of the β1, β2, or β3 families. Only mAbs to CD18 (β2) were inhibitory (36). Likewise, a mAb to CD18 blocked the ability of TNF to lower cAMP in PMN adherent to FBS-coated plastic. The specificity of the effect of anti-CD18 mAb was demonstrated by using a control mAb that binds to the CD11b component of the same integrin heterodimer. The anti-CD11b mAb blocked neither the TNF-induced respiratory burst (reference 36, confirmed here [not shown]) nor the TNF-induced fall in cAMP (Fig. 3). In four such experiments, TNF treatment led to a 33% fall in cAMP in cells not exposed to mAb; anti-CD18 mAb reduced this to a 5% fall. By analysis of variance, the difference between the inhibitory effect of anti-CD18 mAb and the lack of inhibitory effect of anti-CD11b mAb was significant ($P < 0.005$).

A critical role for CD11/CD18 molecules was confirmed by testing PMN from a child with leukocyte adhesion deficiency. These PMN lacked any detectable CD11/CD18. Their cAMP fell by only 4.8% during incubation with TNF on FBS-coated plastic. In contrast, the same cells could lower their cAMP in response to a CD18-independent adhesion event, as will be shown below.

Effect of cAMP-elevating Agents on the Respiratory Burst

The above results established that cAMP began to fall in TNF-treated, adherent PMN before the onset of the respiratory burst. To determine if the fall in cAMP was consequential for the subsequent release of H_2O_2, we tested the effect of adding agents that elevate cAMP in PMN (5, 29, 30), with particular emphasis on the timing of their addition. A previous report demonstrated that dibutyryl cAMP could suppress the brief respiratory burst induced by TNF in suspended PMN (60). After this study was completed, similar findings were described in adherent PMN (29). However, there have apparently been no reports of the effect on the TNF-induced respiratory burst of various cAMP-elevating drugs tested individually and in combination, or added at different times. Fig. 4 shows the complete, long-lasting, entirely synergistic inhibition of the TNF-induced respiratory burst afforded by the phosphodiesterase inhibitor IBMX in conjunction with either the lipid permeable cAMP analog dibutyryl cAMP or the adenylyl cyclase-activating diterpene, forskolin.

It was particularly striking that the combination of IBMX and dibutyryl cAMP inhibited the TNF-induced respiratory burst completely if added any time during the lag period, even as late as 2 min before the onset of H_2O_2 release, whereas addition of the same agents as early as 3 min after the onset of H_2O_2 release was scarcely inhibitory (Fig. 5).

Inhibition by the cAMP-elevating agents was nontoxic and relatively specific, because the respiratory burst in response to PMA was unaffected (Figs. 4 and 5); there was no synergistic interaction between IBMX and dibutyryl cGMP (Fig. 4); and IBMX plus dibutyryl cAMP did not inhibit the TNF-

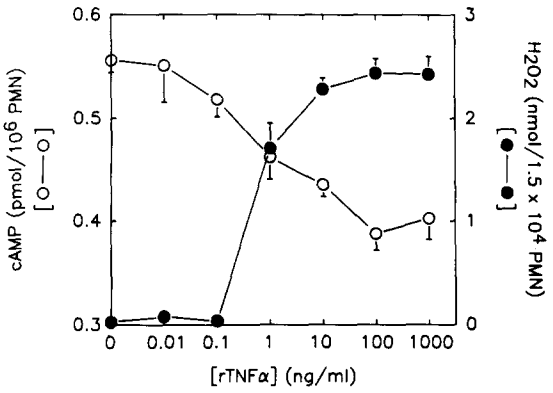

Figure 2. Concentration dependence of TNF's ability to lower cAMP (*open circles*) and stimulate H_2O_2 release (*closed circles*). Both tests were made at the same time in the same experiment using PMN in FBS-coated polystyrene wells. Results are means ± SEM at 45 min for one of two similar experiments.

*Table II. Combined Effects of TNF and Adherence to Fibrinogen-coated Plastic on cAMP Levels in PMN**

Status	TNF	cAMP
	ng/ml	*pmol/10⁶ PMN*
Suspended	0	0.500 ± 0.024‡
Suspended	100	0.442 ± 0.016
Adherent	0	0.429 ± 0.005
Adherent	100	0.280 ± 0.037

* Results are for 45-min incubations at 37°C.
‡ Means ± SEM for one of two similar experiments, each in triplicate. Time zero values averaged 0.401 ± 0.020 pmol cAMP/10^6 PMN.

induced respiratory burst if added after the burst had already commenced (Fig. 5).

These results suggested that a fall in cAMP was a prerequisite for the TNF-induced respiratory burst to begin, but that once the burst had started, the level of intracellular cAMP was no longer consequential.

Effect of Known Respiratory Burst Inhibitors on cAMP Levels

The dependence of the inhibitory effect of cAMP-elevating drugs on the time of their addition, and the selectivity of their inhibition for the respiratory burst induced by TNF but not by PMA, are characteristics previously demonstrated for two other sets of compounds of widely different structures: the autacoid adenosine and its analogues (16), and cytochalasins (34), inhibitors of actin polymerization. Adenosine elevates cAMP in several cell types, but this has not been observed in PMN, unless they were also treated with an inhibitor of cAMP phosphodiesterase (12). In the present experiments, inhibition of the TNF-induced respiratory burst by each of these agents was confirmed (not shown) and compared with their effects on cAMP levels. Inhibitors of phosphodiesterase were not used in these experiments. 2-Chloroadenosine led to an increase in cAMP in PMN. The increase was greater in adherent than suspended PMN and in the presence of TNF (Fig. 6). Likewise, in the presence of DHCB

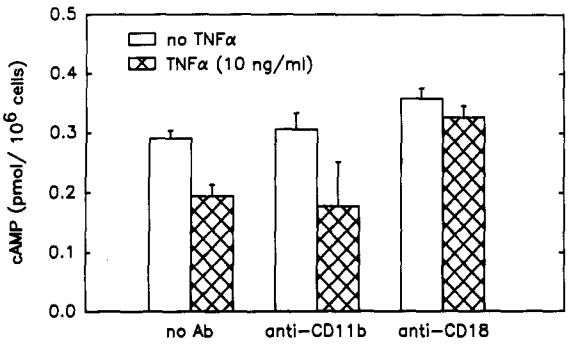

Figure 3. Ability of anti-CD18 mAb to block the fall in cAMP in TNF-treated, adherent PMN. PMN were pretreated in suspension for 90 min at 37°C with no mAb or with OKM1 anti-CD11b IgG2b or IB4 anti-CD18 IgG2a (each 30 μg/ml). The PMN were then incubated for a further 45 min in FBS-precoated wells with or without TNF (10 ng/ml) and sufficient mAb to maintain the same concentrations as during the preincubation. Results are means ± SEM for triplicates from one of four similar experiments.

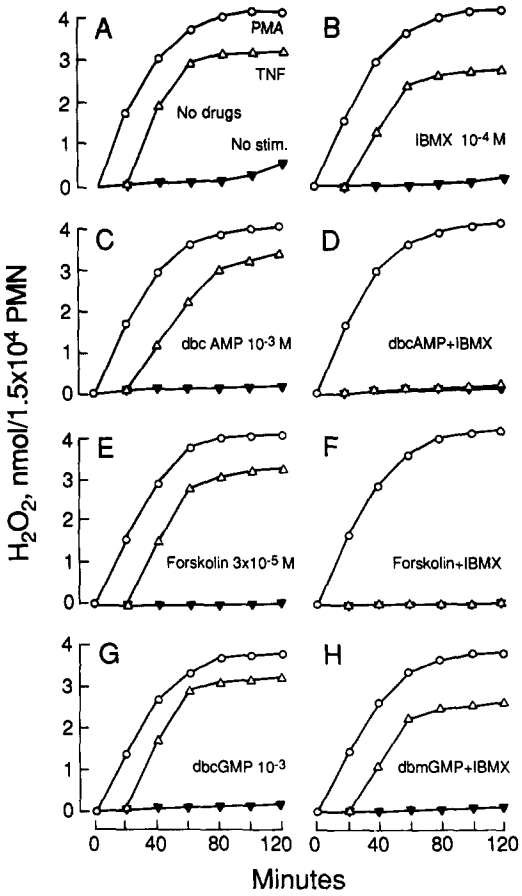

Figure 4. Selective suppression of the TNF-induced respiratory burst by agents that elevate cAMP. H_2O_2 release from PMN on FBS-coated Primaria plates was measured with no secretagogue (no stim.; *inverted triangles*) or with PMA (*circles*) or TNF (*upright triangles*) (100 ng/ml each). Cells received (*A*) no other drugs, or were treated with the indicated concentrations of (*B*) IBMX, (*C*) dibutyryl cAMP, (*D*) dibutyryl cAMP plus IBMX, (*E*) forskolin, (*F*) forskolin plus IBMX, (*G*) dbcGMP, or (*H*) dbcGMP plus IBMX. Results are means of triplicates; SEM were <5%. Note that the response to PMA was unaffected, whereas induction of the respiratory burst by TNF was synergistically suppressed in *D* and *F*, but not *H*.

(5 μg/ml), cAMP in TNF-treated cells adherent to FBS-coated wells fell only 13.8 ± 6.6% (mean ± SEM of the percent fall in three experiments), compared to a fall of 35.2 ± 2.4% without DHCB in the same experiments (Table III). By analysis of variance, the inhibitory effect of DHCB was significant ($0.01 < P < 0.025$).

Thus, 2-chloroadenosine elevated cAMP under the same conditions in which it inhibited the TNF-induced respiratory burst. Moreover, the ability of PMN to polymerize actin appeared to be required for TNF to induce a fall in cAMP.

Kinetics of TNF-induced Spreading of PMN on Protein-coated Surfaces

The ability of an inhibitor of actin polymerization to diminish the fall in cAMP directed our attention to the effects of TNF and cAMP on the shape of adherent PMN and their assembly of actin filaments.

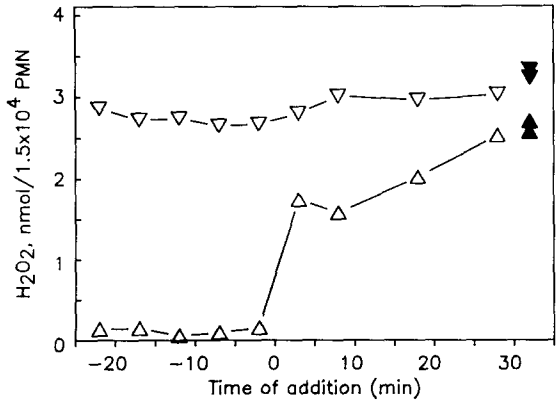

Figure 5. IBMX plus dibutyryl cAMP inhibit the TNF-induced respiratory burst only if added before its onset. Open symbols indicate H_2O_2 released at 60 min by nine different sets of PMN (each in triplicate) in which the time of addition of IBMX (0.1 mM) plus dibutyryl cAMP (1 mM) was varied. The abscissa indicates the time of addition relative to the end of the lag period, which is set at 0. Negative numbers are minutes before the end of the lag period; positive numbers are minutes after the end of the lag period. Cells were treated with either PMA (*inverted triangles*) or TNF (*upright triangles*) (100 ng/ml each). Solid symbols are corresponding values from duplicate sets of PMN stimulated in the absence of IBMX and dibutyryl cAMP. In each of these two control sets treated with TNF, the respiratory burst began 22 min after the addition of TNF. A repeat experiment gave nearly identical results.

Shape changes were monitored simultaneously with the respiratory burst and changes in cAMP (compare Fig. 1 with Fig. 7). PMN fixed in suspension at 37°C were spherical and refractile by phase contrast microscopy (not shown). When ice-cold PMN were added to prewarmed buffer (37°C) in vessels having glass or plastic surfaces coated with FBS, nearly half the cells became loosely attached within 2 min. Cells were considered loosely attached when they became phase-dark and irregular in outline, but did not spread, and resisted dislodgment when the vessel was jarred. Such cells were not tightly attached, because almost all of them came off if the plate was flicked and flooded three times (Table IV). In the absence of TNF, loose attachment of about half the

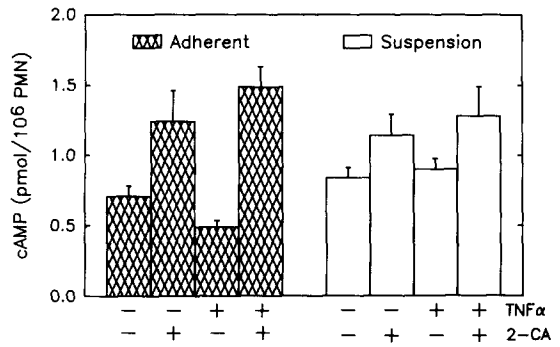

Figure 6. Ability of 2-chloroadenosine (2-CA; 30 μM) to elevate cAMP in PMN by itself and in conjunction with TNF (100–300 ng/ml). Results pertain to 45-min incubations and are means ± SEM for triplicates pooled from each of two experiments. No phosphodiesterase inhibitors were added.

*Table III. Effects of DHCB on the TNF-induced Fall in cAMP in Adherent PMN**

DHCB	TNF	cAMP	Percent decline‡
μg/ml	*ng/ml*	*pmol/10^6 PMN*	
0	0	0.514 ± 0.143§	–
0	100	0.338 ± 0.103	35.2 ± 2.4
5	0	0.506 ± 0.144	–
5	100	0.425 ± 0.120	13.8 ± 6.6

* PMN were in contact with FBS-coated plastic for 45 min at 37°C.
‡ TNF-treated set compared with control set immediately preceding. Results are means ± SEM from individual percentages of decline in each of three experiments.
§ Means ± SEM of mean results from three experiments, each in triplicate.

cells was a stable property of the culture throughout a 90-min period of observation (Fig. 7 *A*).

In contrast, when TNF was added, 95% of PMN attached within 20 min (Fig. 7 *B*). After a 15-min lag, TNF-treated PMN began to spread extensively, increasing their longest diameter from ~12 to ~40 μm (Figs. 7 *B*, and 8 *A*). Recruitment into the population of spread cells continued until 90 min, as long as the respiratory burst lasted in the population as a whole. At 30 min, most spread cells were crescentic, with dendritic pseudopodia (Fig. 8 *A*). By 60 min, most were discoid, with ruffled margins (Fig. 8 *C*). TNF-treated, well-spread cells were tightly attached, in that they resisted forceful dislodgment (Table IV). Results shown in Figs. 7 and 8

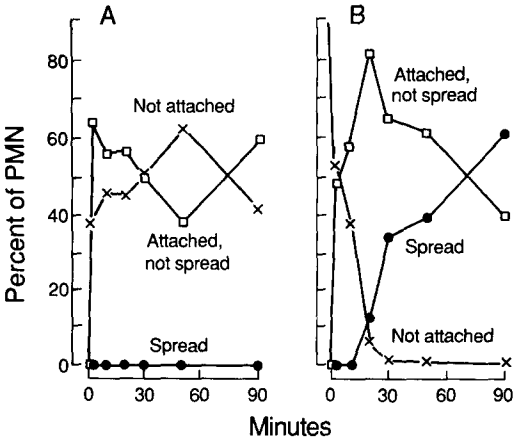

Figure 7. Time course of morphologic changes. PMN were studied in the same experiment illustrated in Fig. 1. (*A*) Unstimulated PMN. (*B*) PMN exposed to TNF (100 ng/ml). At the indicated times, PMN in separate plates were fixed with 1.25% glutaraldehyde at 37°C, and 200 cells per condition were scored as being phase-bright, spherical, and dislodgeable by horizontal acceleration of the plate (not attached; *crosses*); similar in diameter to the first group (not more than twofold greater) but phase-dark, round or slightly irregular in outline, and not dislodgeable (attached, not spread; *squares*); or two- to fourfold larger in diameter than the first group, phase-dark, highly irregular in outline, and not dislodgeable (spread; *circles*). The sum of the three experimentally determined proportions totaled 100 at each time point. The onset of spreading of TNF-treated cells was estimated to occur at 15 min by back-extrapolation of the maximal rate to the baseline, preceding the onset of the TNF-induced respiratory burst in the same experiment by 22 min. A repeat experiment gave nearly identical results.

*Table IV. PMN Adherence**

Exp.	Drugs	30 min		60 min	
		No stimulation	TNF (10 ng/ml)	No stimulation	TNF
		μg cell protein/well (means ± SEM for triplicates)			
A	None	1.79 ± 0.15	6.53 ± 0.08	1.22 ± 0.14	12.10 ± 0.66
	IBMX (*0.1 mM*) + dbcAMP (*1 mM*)			1.91 ± 0.16	3.80 ± 0.51
	IBMX + Forskolin (*30 μM*)			1.41 ± 0.53	7.20 ± 2.40
B	None			1.30 ± 0.32	10.02 ± 0.07
	DHCB (*5 μg/ml*)			−0.25 ± 0.06	8.94 ± 0.19

* The results in this table reflect tight adherence of PMN (resistance to forceful dislodgment) rather than cell spreading. 10^5 PMN in 100 μl H_2O_2 reaction mix (used for comparability to other assays and as a peroxide-scavenging system) were added to FBS-precoated 6-mm-diam wells in polystyrene tissue culture plates and incubated at 37°C in air. After 30 or 60 min, the plates were flicked dry, then immersed in a 1-liter beaker of NaCl (300 mosM) at 37°C. This cycle was repeated until the plates had been flooded three times and flicked four times. Protein contributed by tightly adherent cells was then measured as described in Materials and Methods. Recovery of 12 μg protein implies that 100% of the cells were tightly adherent.

were the same when the surfaces were coated with fibrinogen rather than FBS (not shown).

Elevation of cAMP Inhibits TNF-induced Cell Spreading and Assembly of Actin-containing Microfilaments

The fall in cAMP in TNF-treated, adherent PMN began at about the same time as cell spreading. Both events preceded the respiratory burst. Agents that elevated cAMP prevented the respiratory burst only if they were added during the period when cell spreading normally began. Thus it was of interest to test the effect of IBMX plus dibutyryl cAMP on TNF-induced spreading, and to determine whether the effect of these agents depended on the time of their addition. The cAMP-elevating agents did not prevent TNF-treated PMN from becoming loosely adherent (phase-dark and irregular in outline, Fig. 8, *B* and *D*). In fact, PMN treated with IBMX and dibutyryl cAMP remained partially resistant to forceful dislodgment (Table IV). However, the cAMP-elevating agents completely blocked the cell spreading normally seen in response to TNF (compare Fig. 8, *A* and *B* at 30 min, or Fig. 8, *C* and *D* at 60 min).

As with inhibition of the respiratory burst, the cAMP-elevating agents only inhibited cell spreading if added before spreading started. Thus, a 30-min exposure to IBMX plus dibutyryl cAMP, beginning 30 min after addition of TNF, did not reverse spreading that had already commenced (compare Fig. 8 *E* and *C*). Dependence of inhibition on time of addition indicated that the inhibitors were nontoxic. Lack of toxicity was further indicated by the reversibility of inhibition of cell spreading. Thus, when IBMX plus dibutyryl cAMP were added along with TNF for the first 30 min, and the coverslips bearing PMN were then transferred into drug-free buffer, the PMN resumed spreading, and attained the crescentic stage 30 min later (Fig. 8 *F*). Finally, as with the PMA-induced respiratory burst, IBMX plus dibutyryl cAMP had no effect on PMA-induced cell spreading (not shown).

At 30 and 60 min, TNF-induced cell spreading was associated with formation of an extensive lattice of actin-containing filaments throughout the cytoplasm (Fig. 9, *A* and *C*). A 30-min exposure of TNF-treated PMN to IBMX plus dibutyryl cAMP prevented the formation of these structures. Instead, fluorescence was confined to bright rings at the cell periphery (Fig. 9 *B*). By 60 min of exposure to cAMP-elevating drugs, binding of rhodamine-phallacidin by many of the TNF-treated PMN was no longer perceptible (Fig. 9, *D* and *E*). As with cell spreading, inhibition of actin filament assembly required that the cAMP-elevating drugs be added before actin reorganization commenced. Thus, the drugs had no effect on the assembly of actin-rich structures in PMN that had been treated with TNF for 30 min before the drugs were added (Fig. 9 *F*). Likewise, the inhibitory effect of these agents on actin reorganization was fully reversible (Fig. 9 *G*), and did not extend to PMA-treated PMN (not shown). The same cAMP-elevating drugs had no effect on the staining of TNF-treated PMN with antitubulin antibody (not shown).

Thus, a fall in cAMP appeared necessary for the onset of TNF-induced cell spreading and actin reorganization. Once cell shape had begun to change in response to TNF, the level of cAMP no longer appeared consequential.

Adherence to Uncoated Plastic Mimics the Combined Effects of TNF and Integrins

Uncoated glass or polystyrene surfaces trigger the respiratory burst in PMN without the participation of CD11/CD18 (β2) integrins (36, 45). Likewise, when plated on uncoated plastic, PMN spread rapidly (not shown), and cAMP fell markedly, without a requirement for TNF (Table V). Proof that uncoated plastic itself can induce a fall in cAMP without the participation of CD11/CD18 integrins was provided by PMN deficient in CD11/CD18 (β2) integrins. When the latter cells were plated on uncoated plastic without TNF, their cAMP fell 29.4% by 45 min.

Thus, a sustained decline in cAMP, cell spreading, and a respiratory burst all required exposure, either to a protein-coated surface together with TNF, or alternatively, to uncoated polystyrene alone.

Discussion

Ligation of TNF receptors and engagement of CD11/CD18 (β2) integrins by protein-coated surfaces interacted synergistically in PMN to bring about a sustained fall in cAMP, spreading of the cells, and reorganization of actin. Close

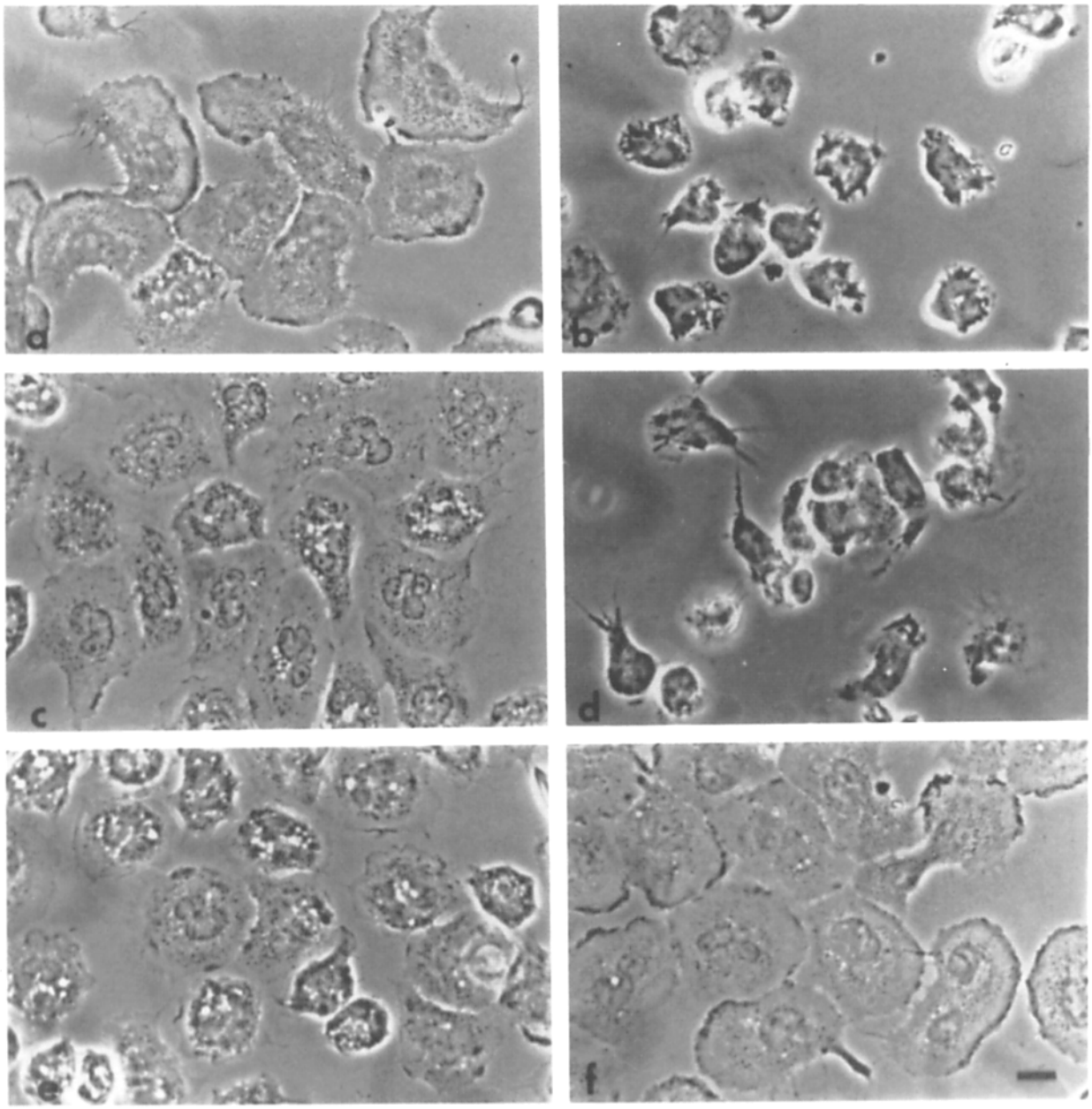

Figure 8. TNF-induced cell spreading and its prevention by agents that elevate cAMP. In all panels, TNF (10 ng/ml) was added at time 0 to PMN on FBS-coated glass coverslips. (*A*) 30-min incubation without cAMP-elevating drugs. (*B*) 30-min incubation with dibutyryl cAMP (1 mM) plus IBMX (0.1 mM). (*C*) 60-min incubation without cAMP-elevating drugs. (*D*) 60-min incubation with dibutyryl cAMP + IBMX. In *E* and *F*, the coverslips were removed from the wells at 30 min, rinsed, and transferred to a fresh well for a further 30 min incubation. In *E*, only the second well contained dibutyryl cAMP + IBMX. In *F*, only the first well contained dibutyryl cAMP plus IBMX. Thus, the cAMP-elevating agents could prevent spreading (compare *B* with *A*, and *D* with *C*), but not if added after spreading commenced (*E*), and the inhibition of spreading was reversible (*F*). TNF-treated PMN were consistently crescentic (*A*) before becoming disk-like (*C*). In contrast, the spreading induced by PMA was disk-like from the outset (not shown). Untreated PMN remained spherical (not shown). Bar, 10 μm.

connections among the fall in cAMP, the reorganization of actin, and the subsequent respiratory burst were evident from the coordinate inhibition of all three processes by five structurally diverse drugs, an anti-CD18 mAb, and genetic deficiency of CD18.

These observations make several new points. The ability of PMN to spread is central to their physiologic functions, including adherence to endothelium, emigration from blood vessels, and chemotaxis into tissues. TNF appears to be a major physiologic activator of neutrophils (29, 34, 45), and increases F actin in PMN (4). Yet the spreading of PMN in response to TNF has apparently not been analyzed previously. Nor has it been shown before that TNF affects the levels of cyclic nucleotides in PMN. Indeed, acute effects of TNF on cellular cAMP have previously been reported only in fibroblasts, where cAMP rose (61). Elevations in cAMP

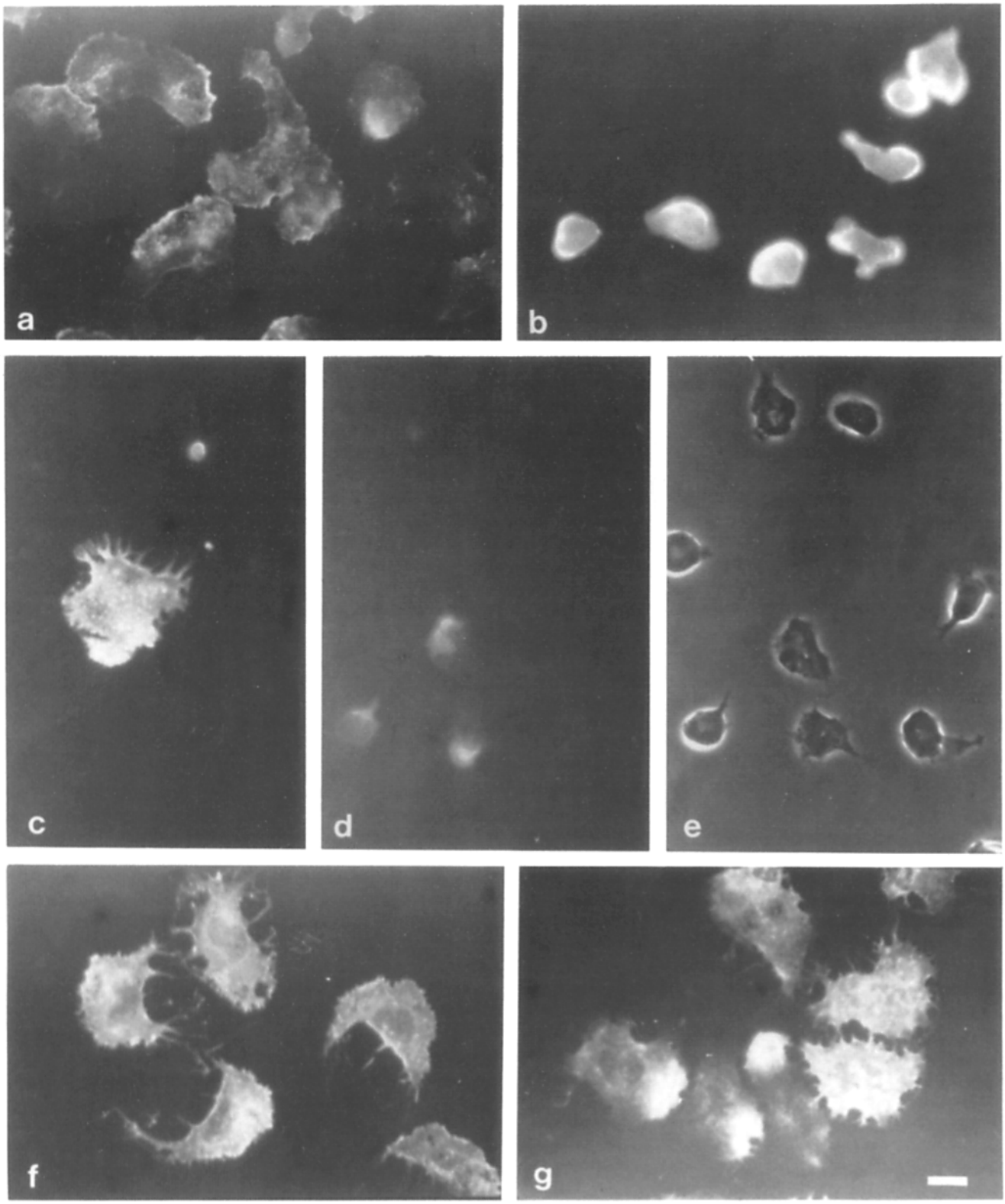

Figure 9. Reorganization of actin in TNF-treated PMN and its inhibition by cAMP-elevating agents. All panels except *E* are fluorescent photomicrographs of cells stained with rhodamine-phallacidin; *E* is a phase-contrast image of the same field as in *D*. TNF (10 ng/ml) was added at time 0 to PMN on FBS-coated glass coverslips. In *B*, *D*, and *E*, the wells also contained dibutyryl cAMP (1 mM) plus IBMX (0.1 mM). The incubations were terminated at 30 (*A* and *B*) or 60 min (*C–E*). By 60 min of exposure to the cAMP-elevating agents, many cells displayed no detectable fluorescence (compare *D* with *E*). In *F* and *G*, the coverslips were removed from their primary wells at 30 min, rinsed, and transferred to secondary wells for a further 30 min incubation. In *F*, only the primary well contained dibutyryl cAMP plus IBMX. In *G*, only the secondary well contained dibutyryl cAMP plus IBMX. Thus, the cAMP-elevating agents could prevent assembly of actin-containing filaments, but not if added after assembly commenced (*G*), and the inhibition of assembly was reversible (*F*). Pattern of fluorescence in PMN not given TNF resembled that in *B* (not shown). Bar, 10 μm.

*Table V. Combined Effects of TNF and Adherence to Uncoated Plastic on cAMP Levels in PMN**

Status	TNF	cAMP
	ng/ml	*pmol/10⁶ PMN*
Suspended	0	0.517 ± 0.011‡
Suspended	100	0.470 ± 0.017
Adherent	0	0.257 ± 0.070
Adherent	100	0.224 ± 0.011

* Results are for 45-min incubations at 37°C.
‡ Means ± SEM for one of four similar experiments on uncoated plastic, each in triplicate.

can cause cells to round up (27, 53); the present observations may be the first to suggest that a fall in cAMP can be permissive for cell spreading. Most important, the present study affords one of the first examples of the ability of integrins to affect the level of an intracellular second messenger.

These findings contribute to an emerging picture of the activation of lymphohematopoietic cells through synergistic interactions involving integrins on the one hand, and receptors for antigens, cytokines or microbial products on the other hand. For example, engagement of β1 or β2 integrins synergistically enhances T cell proliferation induced through the antigen receptor (33, 54; Shimizu, Y., G. A. van Seventer, K. J. Horgan, and S. Shaw. 1990. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 4:A1791). Likewise, adherence interacts synergistically with bacterial products to induce monocytes to synthesize cytokines (50). The present results with PMN differ from those with mononuclear cells in that the PMN response is faster and does not depend on protein synthesis (34).

It is not clear how a fall in cAMP prepared PMN for the onset of the respiratory burst. One possible mechanism is that a fall in cAMP may alter a key function of TNF receptors or integrins. For example, it has been shown that elevation of cAMP inhibits the binding function of CD11a/CD18 in T lymphocytes (18). Thus, a fall in cAMP might enhance the binding (or signaling) functions of CD11/CD18 molecules in PMN. This would presumably result from altered phosphorylation of the integrins themselves (6, 8, 55) or of an associated molecule. It remains to be seen if TNF receptors (31, 43, 48) or molecules closely associated with their function (59) undergo phosphorylation. If so, the same considerations could apply.

Alternatively, the critical effect of the fall in cAMP could be to facilitate TNF-induced actin polymerization. Actin within PMN is a substrate of cAMP-dependent protein kinase (22), and microinjection of fibroblasts with the catalytic subunit of cAMP-dependent protein kinase inhibits actin polymerization (27). Other substrates of cAMP-dependent protein kinases that are relevant to control of the cytoskeleton include myosin light chain kinase, which can control actin polymerization (27); vimentin (7, 28); possibly talin (53); and a Na^+, K^+-ATPase (52), which may be the enzyme responsible for the intracellular alkalinization that accompanies the spreading of fibroblasts (44).

The next question to consider is why actin-dependent cell spreading appears to be required for the onset of the TNF-induced respiratory burst. The electron transport chain that produces superoxide is assembled in the plasma membrane by recruiting components from the specific granules and cytosol (1). The intracellular traffic required to bring these components together may be facilitated by a redistribution of subcortical actin from the organelle-excluding hyaline ectoplasm into adhesion placques and bundles (13). Alternatively, the actin-based cytoskeleton may contribute directly to the assembly of a functioning oxidase. Thus, the 47-kD phosphoprotein component of the respiratory burst oxidase (itself a substrate of cAMP-dependent protein kinase [26]), a portion of the cytochrome b_{558}, and most of the oxidase enzymatic activity were found to associate with the detergent-insoluble cytoskeletal fraction of PMN (Babior, B. M., J. T. Curnutte, and N. Okamura. 1990. *Blood.* 72(Suppl. 1):141.) In suspended PMN, an oscillatory respiratory burst could be induced whose periodicity coincided with oscillatory actin polymerization and cell extension (57). Finally, TNF receptors may need to associate with the cytoskeleton in order to activate the oxidase.

A brief elevation in cAMP in suspended PMN follows exposure to formylated peptides and has been studied extensively (3, 58); the physiologic consequences of this response are unknown. In contrast, a sustained decline in cAMP in PMN has not been observed previously. Our studies do not define the biochemical basis of this response. It is well known that the same hormone or autacoid can affect both adenylyl cyclase and cAMP phosphodiesterase, leading to a net fall in cAMP. Examples are insulin (14, 21, 41, 42), insulin-like growth factor I (41), progesterone (41), and muscarinic cholinergics (23). In such systems, changes in cAMP of a magnitude similar to those seen here were considered critical to hormone action. Moreover, progesterone and insulin can interact synergistically to activate phosphodiesterase and lower cAMP in *Xenopus* oocytes (41). This provides a precedent for the synergistic interaction of distinct types of receptors in lowering cAMP in PMN. Thus, engagement of TNF receptors and integrins could lower cAMP in PMN by activating cAMP phosphodiesterase, inhibiting adenylyl cyclase, and/or stimulating cAMP secretion. Asynchronous onset and offset of these processes could explain the three phases observed in the cAMP levels of PMN making contact with protein-coated surfaces. A precipitous drop in cAMP in the first 2 min was independent of TNF, and thus has not been emphasized in this report. In TNF-treated cells in the middle phase (~10–90 min), cAMP was low in the absence of pharmacologic inhibitors, but did rise above baseline in the presence of a phosphodiesterase inhibitor (unpublished observations). This could be explained by predominant activation of cAMP phosphodiesterase, accompanied by a quantitatively less important activation of adenylyl cyclase. A return of phosphodiesterase activity to normal could then explain the last phase, in which cAMP levels returned to baseline. Direct assays of these enzymes will be required to test these inferences.

Colony-stimulating factor for granulocytes/macrophages resembles TNF in its ability to trigger a respiratory burst in adherent PMN (35). Adenylyl cyclase was inhibited by 30–40% after a 10-min incubation of PMN in CSF-GM (9). However, cAMP did not fall. The conditions of the experiments were not conductive to adherence of the cells to matrix protein–coated surfaces (9). It would be of interest to test whether cAMP would fall in response to CSF-GM (or other physiologic agonists) under the same conditions in which these agents trigger a prolonged respiratory burst (34, 35).

The sequence of rapid attachment and subsequent spreading, the ability of cytochalasins to inhibit spreading but not adherence, and the morphologic appearance of the microfilament network in spread PMN in response to TNF were all strikingly similar to what Southwick et al. have recently described for PMN on uncoated plastic surfaces (49). Such surfaces are themselves potent, CD11/CD18-independent stimuli of the respiratory burst (36). Indeed, cAMP fell markedly in PMN plated on uncoated plastic, without a requirement either for TNF or for CD11/CD18 ($\beta 2$) integrins. Uncoated plastic seems to mimic or bypass combined signals that are otherwise required from adhesion receptors and peptide receptors when cells are plated on more physiologic surfaces.

In conclusion, the observations reported here indicate that integrins can interact with cytokines to influence cell shape and function by affecting the intracellular level of cAMP.

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