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HOST DEFENSE AGAINST OPPORTUNIST MICROORGANISMS FOLLOWING TRAUMA

Annual Report

June 30, 1989

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<p>Prostaglandins of the E series inhibit major effector functions of polymorphonuclear leukocytes (PMNs) by elevating intracellular cyclic-3',5'-adenosine monophosphate (cAMP). The present study investigated the involvement of this mechanism in the bactericidal defect of PMNs induced by thermal injury in a guinea pig model. Peripheral and peritoneal exudate PMNs harvested from thermally injured guinea pigs at one or two days postburn had decreased bactericidal activity against <i>Pseudomonas aeruginosa</i> and a marked increase in cAMP content. Production of prostaglandin E₁ (PGE₁) by these cells in the absence of exogenous PMN activators was also increased. Treatment of PMNs in vitro or in vivo with nonsteroidal anti-inflammatory drugs (NSAIDs; indomethacin, ibuprofen, and piroxicam) restored bactericidal activity to normal and concomitantly reduced cAMP content and PGE₁ production. A concomitant reduction in cAMP content and PGE₁ production was also observed.</p> <p>continued on next page</p>			
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served as bactericidal activity of PMNs returned to normal under natural conditions during four to seven days postburn. The enhancement of PMN bactericidal activity mediated by NSAIDs was fully counteracted by purified PGE₁, theophylline, and by cAMP itself. These results suggest that the bactericidal defect of PMNs induced by thermal injury is related to elevation of cAMP and that PGE₁ plays a significant role in this phenomenon.

Thermal injury

FOREWORD

In conducting the research described in this report, the investigators adhered to the Guide for the Care and Use of Laboratory Animals, prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 86-23, Revised 1985). Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

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TABLE OF CONTENTS

	<u>Page</u>
Foreword	3
List of Figures and Tables	5
Body of Report	6
Statement of the Problem under Study	6
Methods	7
Results	13
Discussion and Conclusions	29
Reference List	36

LIST OF FIGURES AND TABLES

	<u>Page</u>
Figure 1. Effect of varying the bacteria to leukocyte ratio on the postburn bactericidal defect of PMNs.	14
Figure 2. Effect of thermal injury on O_2^- production by PMNs in response to FMLP.	15
Figure 3. Effect of NSAIDs on the bactericidal activity of PMNs from thermally injured animals.	17
Figure 4. Effect of NSAIDs on the bactericidal activity of PMNs from normal animals.	18
Figure 5. Effect of various concentrations of NSAIDs on the bactericidal activity of PMNs from thermally injured animals.	19
Figure 6. Effect of PGE_1 on the bactericidal activity of PMNs from thermally injured animals treated in vitro with piroxicam.	21
Figure 7. Effect of PGE_1 on the bactericidal activity of PMNs from thermally injured animals treated in vivo with piroxicam.	22
Figure 8. Effect of PGE_1 and PGE_2 on the bactericidal activity of PMNs from normal animals.	23
Figure 9. Effect of cAMP and theophylline on the bactericidal activity of PMNs from thermally injured animals treated in vitro or in vivo with piroxicam.	24
Figure 10. Effect of cAMP and theophylline on the bactericidal activity of PMNs from normal animals.	25
Table I. cAMP content of PMNs under various conditions.	27
Table II. Effect of NSAIDs on PGE_1 production by PMNs.	28
Table III. Natural changes in cAMP content, PGE_1 production, and bactericidal activity of PMNs during the first week postburn.	30
Table IV. Postburn changes in cAMP content, PGE_1 production, and bactericidal activity of peritoneal exudate PMNs.	31

BODY OF REPORT

Statement of the Problem under Study

Sepsis continues to be a leading cause of morbidity and mortality in thermally injured patients despite recent improvements in the clinical care of these patients [1]. Susceptibility to bacterial infection is increased because of loss of the protective skin barrier and the profound depression of host defense mechanisms induced by severe injury [reviewed in 2]. Polymorphonuclear leukocytes (PMNs) are critically important for maintaining the sterility of tissue spaces and for facilitating clearance of invading bacteria from circulation [3]. Major effector functions of these cells are markedly reduced by thermal injury including chemotaxis, oxidative metabolism, and bactericidal activity [4-13]. Several studies have suggested that the bactericidal defect of PMNs in thermally injured patients contributes to the development of life-threatening infections [4,6,7]. Despite the probable importance of PMN dysfunction in the predisposition to bacterial infection associated with thermal injury, little is known about the mechanisms responsible for this abnormality.

Recent studies have suggested that PMNs are initially activated in response to thermal injury [12]. However, the biochemical events occurring after these cells are activated that lead to the down regulation of critical effector functions are totally unknown. Studies from our laboratory have suggested that arachidonate metabolites of the cyclooxygenase pathway may be involved in this phenomenon. Using a guinea pig model of thermal injury, we demonstrated that the bactericidal activity of PMNs is depressed in temporal association with increased generation of prostaglandins and thromboxane in transudate at the site of thermal injury [14]. In addition, we showed that selective inhibition of the cyclooxygenase pathway by paren-

teral therapy with NSAIDs corrects the bactericidal defect of PMNs in the thermally injured animals [15].

Prostaglandins of the E series are known to inhibit various effector functions of PMNs including chemotaxis, aggregation, superoxide production, and lysosomal enzyme release [16-23]. These prostaglandins exert their effect by acting as adenylyl cyclase agonists, raising intracellular cyclic-3',5'-adenosine monophosphate (cAMP) to an abnormally high level [16-18,20]. It has been suggested that elevation of cAMP serves as an important signal for the cell to stop functioning, replenish energy supplies, and replace the surface membrane. This normal regulatory mechanism may be called into play in response to the heightened activation of PMNs induced by thermal injury resulting in down regulation of effector functions. The present investigation was undertaken to test this hypothesis. Nonsteroidal anti-inflammatory drugs (NSAIDs), prostaglandins of the E series, theophylline, and cAMP were used as tools to modulate the bactericidal activity of PMNs from thermally injured animals. Through this approach and by measuring the cAMP content of the PMNs under identical conditions, we provide evidence that the bactericidal defect of PMNs induced by thermal injury is related to a marked elevation of cAMP.

Methods

Animals. Male and female Hartley guinea pigs weighing 300 to 350 g were purchased from Murphy Breeding Laboratories, Inc., Plainfield, IN. The animals were housed in separate cages and adapted to the new environment for four to five days. The animals were fed guinea pig chow ad libitum.

Experimental thermal injury. Animals were anesthetized by intraperitoneal injection of 30-33 mg/kg sodium pentobarbital (Butler & Co., Columbus, OH) supplemented with inhaled methoxyflurane (Pitman-Moore, Washington

Crossing, NJ) as needed. Failure to flinch in response to pinching of the skin was used as the endpoint to judge that the animals were adequately anesthetized. Full thickness scald burns covering thirty percent of the total body surface were applied as previously described [14]. Fifteen ml of lactated Ringer's solution (Abbott Laboratories, North Chicago, IL) was administered intraperitoneally before and at 1.5 h after injury. Immediately after injury, the animals were placed on heating blankets (set on low) to reduce heat loss and thereby minimize stress. After the second dose of Ringer's solution, the animals were replaced in their cages. Sham-treated animals were used in some experiments. These animals were anesthetized and resuscitated as described above and immersed in tepid water to simulate burning.

Preparation of PMNs. Animals were anesthetized as described above, and blood for preparation of peripheral PMNs was drawn by cardiac puncture into heparinized syringes (50 units of heparin/ml of blood). Blood from two to five animals in each treatment group was pooled in order to yield sufficient numbers of leukocytes to conduct the experiments. One volume of heparinized blood was mixed with two volumes of 3% dextran (200,000-275,000 mol wt; Sigma Chemical Co., St. Louis, MO) in 0.15 M sodium chloride and allowed to sediment for 90 min at 4°C. For bactericidal assays, PMNs were further purified by centrifugation through lymphocyte separation medium (Organon Teknika Corp., Durham, NC) according to McCarthy et al. [24]. For measurements of cAMP content and PGE₁ production, PMNs were further purified by a minor modification of the method of Zimmerli et al. [25]; this method was used, because final preparations contained no contaminating erythrocytes (an artifactual source of cAMP). Three ml of supernatant from the dextran sedimentation was layered onto 4 ml of a 39%/65% Percoll gradient prepared in Hank's balanced salt solution (HBSS; M.A. Bioproducts,

Walkerville, MD). The gradients were centrifuged at 350 g for 25 min at 4°C. PMNs accumulated at the interface were removed and washed once with HBSS. Following both techniques, erythrocytes were lysed with 0.2% sodium chloride for 20 s; isotonicity was restored by addition of an equal volume of 1.6% sodium chloride. The cells were resuspended in Hank's balanced salt solution containing 0.1% gelatin (HBG) for bactericidal assays and HBSS for measurement of superoxide anion (O_2^-) production. For measurements of cAMP and prostaglandin E_1 (PGE_1) production, PMNs were resuspended in HBSS containing 20 mM HEPES (Sigma Chemical Co.), pH 7.4. Final preparations contained $\geq 85\%$ PMNs and $\leq 15\%$ mononuclear cells.

In some experiments, PMNs were obtained from peritoneal exudates. The cells were elicited by intraperitoneal injection of 10 ml of 15% casein (wt/vol) in sterile 0.15 M sodium chloride, pH 7.0 [26]. The peritoneal cavity was lavaged 18 h later with 50 ml of HBSS. Peritoneal exudates from two to three animals were pooled, and PMNs were purified by centrifugation through lymphocyte separation medium and hypotonic lysis as described above. These preparations contained $>95\%$ PMNs.

Measurement of bactericidal activity. A clinical isolate of *Pseudomonas aeruginosa* strain Wk, was grown for 4 h at 37°C in trypticase soy broth (BBL, Cockeysville, MD). The bacteria were washed and resuspended in 0.01 M phosphate buffered saline, pH 7.4. PMN bactericidal activity was measured by enumerating total surviving bacteria by colony counting [27]. Equal parts of bacteria (5.0×10^8 cfu/ml) and 50% pooled normal guinea pig serum (vol/vol) were incubated for 15 min at 37°C. The bacteria were then washed once and resuspended in HBG. Opsonized bacteria (1.0×10^7 cfu/ml) and 5.0×10^6 leukocytes/ml (bacteria to leukocyte ratio of 2:1) were rotated for 60 min at 37°C. In some experiments, the number of opsonized bacteria was increased to 5.0×10^7 cfu/ml or 2.5×10^8 cfu/ml yielding bacteria to

leukocyte ratios of 10:1 and 50:1 respectively. Fifty μ l samples were removed immediately before incubation and after 15, 30, and 60 min of incubation. The samples were serially diluted in distilled water, and the dilutions were plated on MacConkey agar (Difco Laboratories, Detroit, MI). Percent killing was calculated by the formula $(a-b)/a \times 100$, where a and b were equal to surviving cfu/ml before and after incubation respectively.

Measurement of O_2^- production. A minor modification of the method of Fantone et al. [22] was used to measure O_2^- production by peripheral PMNs. Five hundred μ l of leukocyte suspension (4.0×10^6 cells/ml) was preincubated for 5 min at 37°C with 340 μ l of 0.23 mM ferricytochrome c, 10 μ l of 500 $\mu\text{g/ml}$ cytochalasin B, and 50 μ l of HBSS with and without 1 mg/ml superoxide dismutase (SOD). One hundred μ l of 10^{-4} M formylmethionylleucyl-phenylalanine (FMLP) was then added, and the reactants were further incubated for 20 min at 37°C . The reaction was stopped by the addition of 500 μ l of HBSS containing 1 mg/ml SOD to tubes lacking SOD and 500 μ l of HBSS alone to tubes containing SOD. The tubes were centrifuged at 700g for 5 min at 4°C , and the absorbance of the supernatants was read at 550 nm. The amount of O_2^- produced was calculated from the difference in absorbance of the samples with and without SOD in the reaction using a mM extinction coefficient for cytochrome c of 21.1.

Determination of cAMP content. One ml samples containing 5.0×10^6 leukocytes (untreated or treated with NSAIDs and/or cAMP activators as described below) were boiled for 5 min and then sonicated with a virsonic cell disruptor (Virtis Co., Gardner, NY). The cells were centrifuged at 1,000 g for 10 min at 4°C , and the supernatants were assayed for cAMP using a cAMP radioimmunoassay kit from New England Nuclear (Boston, MA). Samples were acetylated and assayed according to the manufacturer's recommended procedure. Greater than 90% of the cAMP reactivity in PMN supernatants

from normal and injured animals was destroyed by incubation for 30 min at 30°C with 0.0005 units/ml of beef heart phosphodiesterase (Sigma Chemical Co.).

Spontaneous production of PGE₁ by PMNs. One ml samples containing 3.0 x 10⁷ leukocytes (untreated or treated with NSAIDs as described below) were incubated for 15 min at 37°C in the absence of exogenous PMN activators. The cells were centrifuged at 750 g for 3 min at 4°C, and the supernatants were removed. Prostaglandins were extracted from the supernatants by the method of Powell [28] using Sep-Pak C₁₈ cartridges (Waters Associates, Milford, MA). Solvent was evaporated under nitrogen flow, and the dried residue was dissolved in radioimmunoassay buffer. PGE₁ was assayed according to the manufacturer's recommended procedure using a kit from Advanced Magnetics (Cambridge, MA). According to the manufacturer, the antiserum to PGE₁ in the kit was 100% cross reactive with PGE₁, 9% cross reactive with PGE₂, and less than 1% cross reactive with other major arachidonate metabolites. A preliminary experiment was conducted to assess the procedural loss through the extraction procedure. Approximately 10,000 cpm of [³H]PGE₁ (Advanced Magnetics) was applied in duplicate to the cartridges and extracted under identical conditions to the PMN supernatants. The procedural loss (mean ± SEM) was 20 ± 1% based on the recovery of radioactivity as compared with the input. Final PGE₁ concentrations were corrected for this procedural loss.

Preparation of NSAIDs. Indomethacin (Sigma Chemical Co.) and piroxicam (Pfizer, Westpoint, PA) were solubilized in 0.2 M sodium phosphate buffer, pH 7.4, at a concentration of 7.5 mg/ml by dropwise addition of 2 N sodium hydroxide to pH 8.5. The solutions were then titrated back to pH 7.4 with 6 N hydrochloric acid. Ibuprofen (50 mg/ml) was obtained from the Upjohn Co., Kalamazoo, MI. For in vitro experiments, the drugs were diluted to 2

mM in 0.2 M sodium phosphate buffer, pH 7.4, before use. The drugs were prepared fresh daily and used immediately.

Treatment of PMNs with NSAIDs in vitro. Leukocytes (1.0×10^7 cells/ml) were preincubated with indomethacin, ibuprofen, or piroxicam for 15 min at room temperature before addition to the assays. Preliminary experiments demonstrated that preincubation of PMNs from normal or injured animals under these conditions in the absence of NSAIDs had no effect on bactericidal activity, cAMP content, or PGE₁ production. Concentrations of NSAIDs used in the experiments are specified in the Results section.

Treatment of PMNs with piroxicam in vivo. In experiments in which the effects of piroxicam in vivo were determined, injured animals were injected intramuscularly with 15 mg/kg of piroxicam at 3 h postburn. In experiments in which PMNs were harvested at two days postburn, the injured animals were injected intramuscularly with a second dose of piroxicam (15 mg/kg) at approximately 18 h postburn.

Addition of cAMP activators or cAMP to assays. In experiments in which cAMP activators or cAMP were tested, these reagents were added to the assays together with normal PMNs or PMNs from injured animals that had been treated with piroxicam in vitro or in vivo as described above. Piroxicam remained in the assays with the cAMP activators or cAMP. The sources of these reagents were as follows: PGE₁ (Advanced Magnetics), PGE₂ (two different preparations, one each from Advanced Magnetics and Upjohn Diagnostics), theophylline (Sigma Chemical Co.), cAMP (Sigma Chemical Co.), and dibutyryl cAMP (Sigma Chemical Co.). For determination of cAMP content, PMNs (5.0×10^6 cells/ml) were incubated with PGE₁, PGE₂, or theophylline for 2 min at 37°C before boiling. Concentrations of cAMP activators and cAMP used in the experiments are specified in the Results section.

Results

Alterations in bactericidal activity and O_2^- production by PMNs following thermal injury. Our previous studies have demonstrated that the bactericidal activity of peripheral PMNs against *P. aeruginosa* is depressed in thermally injured guinea pigs during the first four days postburn and returns to normal by seven days postburn [14,15]. We have also shown that phagocytosis of *P. aeruginosa* by PMNs from the injured animals in the presence of normal serum occurs normally during this period, suggesting that the bactericidal defect reflects only the efficiency of intracellular killing and not an abnormality in phagocytosis [14,15]. In the present study, we attempted to determine whether the postburn bactericidal defect was affected by varying the ratio of PMNs to bacteria in the assay. In these experiments, peripheral PMNs were harvested from injured animals at one day postburn, the time when bactericidal activity was shown to be maximally reduced in our previous studies [14,15]. Bacteria to leukocyte ratios of 50:1, 10:1, and 2:1 were employed in the assay. The postburn bactericidal defect was demonstrable under all three sets of conditions; however, the defect was greatest at bacteria to leukocyte ratios of 10:1 and 2:1 (Fig. 1). The 2:1 ratio was used in subsequent studies.

To determine if the postburn bactericidal defect was associated with an abnormality in oxidative metabolism, FMLP-induced O_2^- production by PMNs from injured and normal animals was compared. O_2^- production by PMNs from injured animals was reduced during three days postburn and returned to normal by seven days postburn (Fig. 2). These results taken together with the results of our previous studies indicated that bactericidal activity and O_2^- production by PMNs were depressed concomitantly in this experimental model.

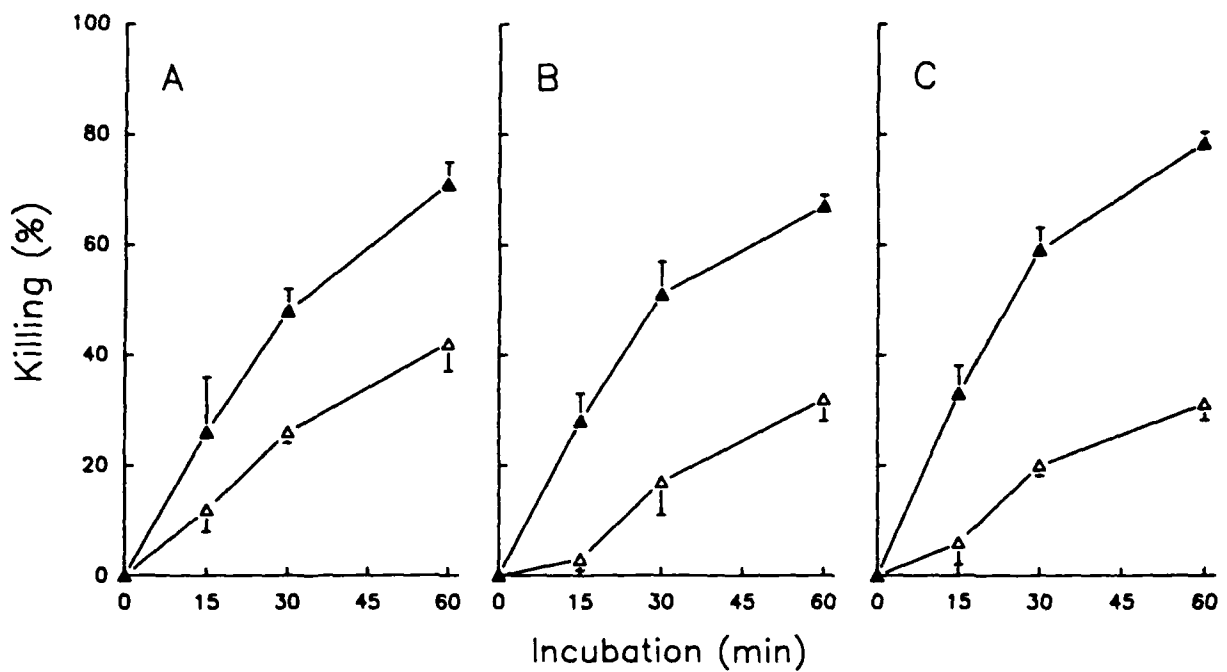


Figure 1. Effect of varying the bacteria to leukocyte ratio on the post-burn bactericidal defect of PMNs. PMNs obtained from injured animals (Δ) at one day postburn and from normal animals (\blacktriangle) were assayed at bacteria to leukocyte ratios of 50:1 (A), 10:1 (B), and 2:1 (C). Data are mean \pm SEM from three separate experiments.

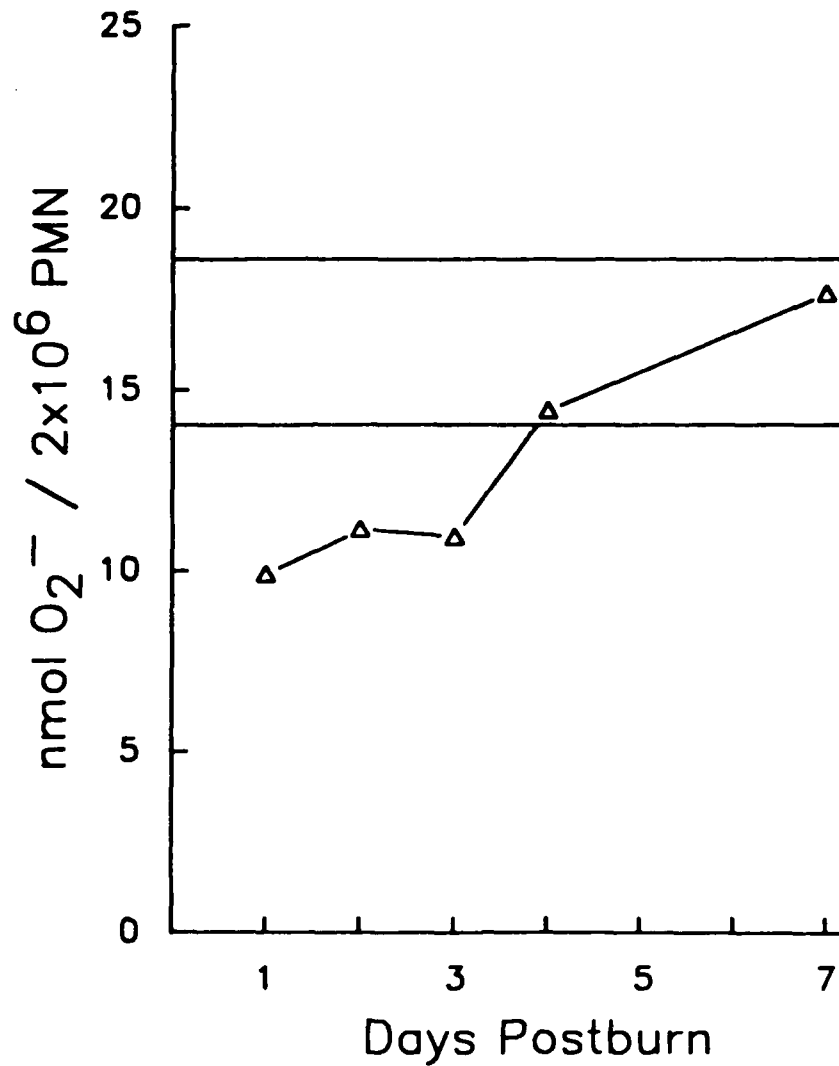


Figure 2. Effect of thermal injury on O_2^- production by PMNs in response to FMLP. The open, boxed area shows the range of O_2^- production by PMNs from sham-treated animals (mean \pm SD) in five separate experiments.

Direct effect of NSAIDs on the bactericidal activity of PMNs. Our previous studies demonstrated that indomethacin, ibuprofen, and piroxicam restored the bactericidal activity of PMNs to normal when administered in vivo to thermally injured animals [15]. To determine if this effect was related to a direct interaction of the NSAIDs with the PMNs, the effect of these drugs on PMN bactericidal activity was determined in vitro. In these and subsequent experiments, PMNs were harvested from injured animals at one or two days postburn. NSAIDs markedly enhanced the bactericidal activity of PMNs from injured animals (Fig. 3) without affecting the bactericidal activity of normal PMNs (Fig. 4).

Additional studies investigated the concentrations of NSAIDs and conditions required for maximal enhancement of the bactericidal activity of PMNs from injured animals. A concentration of 100 μ M NSAID was required to fully restore bactericidal activity to normal; results using piroxicam and indomethacin are shown in Fig. 5. Maximal enhancement of bactericidal activity was achieved when the NSAIDs were preincubated for 15 min at room temperature with the PMNs before assay rather than merely being added to the PMNs without preincubation. Therefore, in all subsequent experiments, PMNs were preincubated with 100 μ M NSAID under these conditions before assay.

Effect of PGE₁ and PGE₂ on the bactericidal activity of PMNs. In the experiments described above, NSAIDs may have enhanced the bactericidal activity of PMNs from injured animals by inhibiting production of E type prostaglandins by these cells. If the observed effects were related to this mechanism, then addition of exogenous PGE₁ or PGE₂ to the assays should counteract the enhancement of bactericidal activity mediated by NSAIDs. To test this hypothesis, PMNs from injured animals were treated with piroxicam in vitro or in vivo, and the bactericidal activity of these

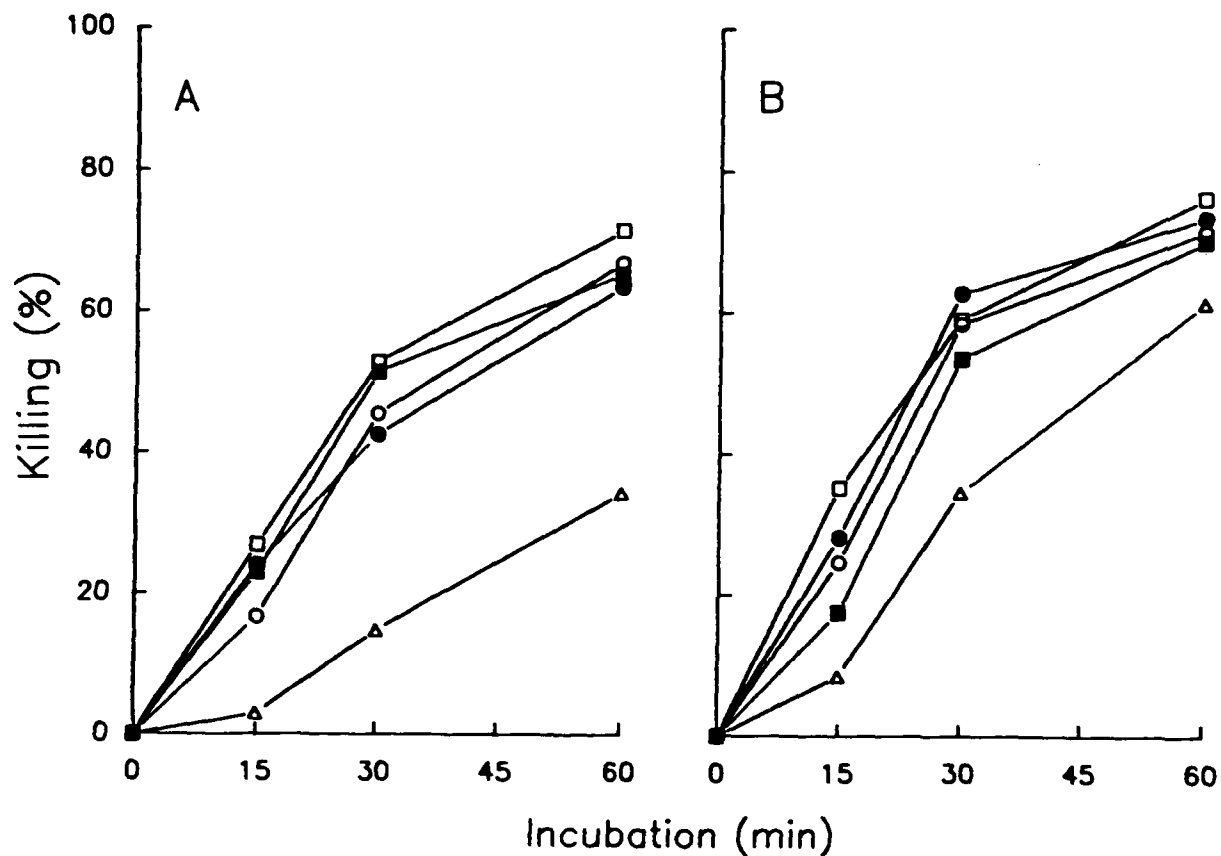


Figure 3. Effect of NSAIDs on the bactericidal activity of PMNs from thermally injured animals. PMNs were harvested from injured animals at one (A) or two (B) days postburn. The cells were preincubated for 15 min at room temperature with 100 μ M indomethacin (○), ibuprofen (■), or piroxicam (●), or received no treatment (△) before addition to the assay. PMNs from normal animals (□) were untreated. Data are from a representative experiment run in duplicate. The difference between duplicates in these and subsequent experiments was usually less than 10%.

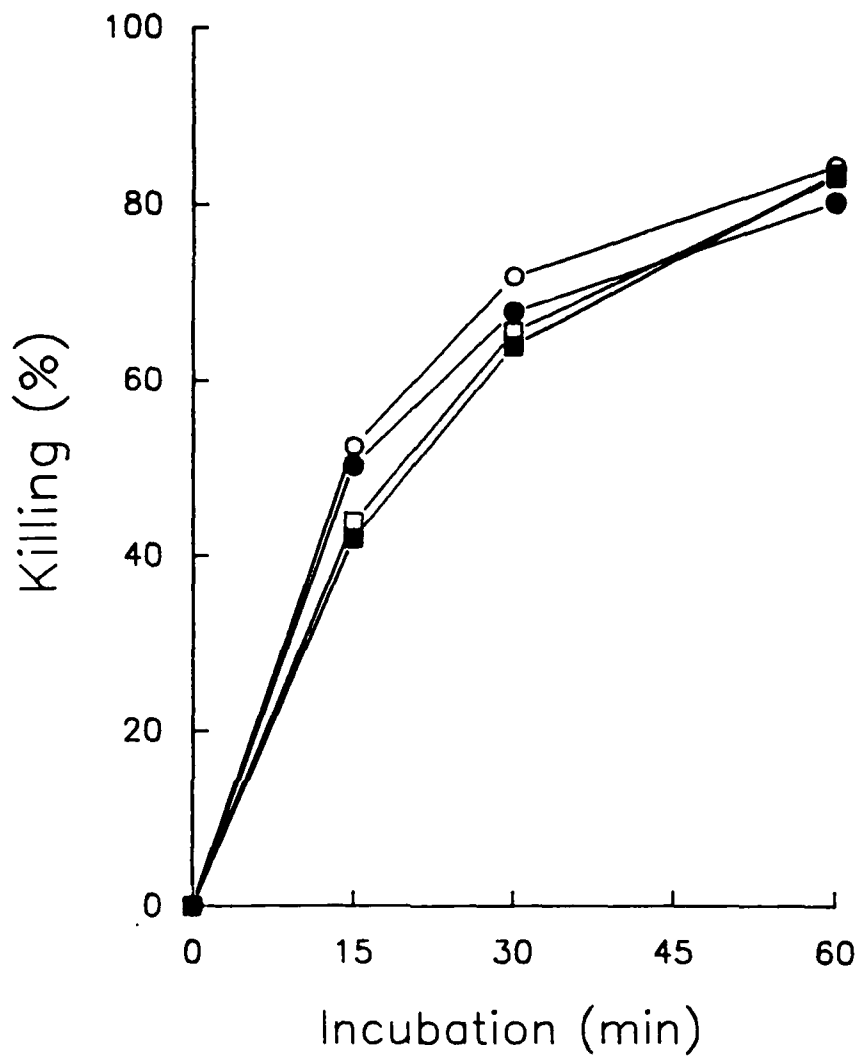


Figure 4. Effect of NSAIDs on the bactericidal activity of PMNs from normal animals. Normal PMNs were preincubated for 15 min at room temperature with 100 μ M indomethacin (O), ibuprofen (■), or piroxicam (●), or received no treatment (□) before addition to the assay. Data are from a representative experiment run in duplicate.

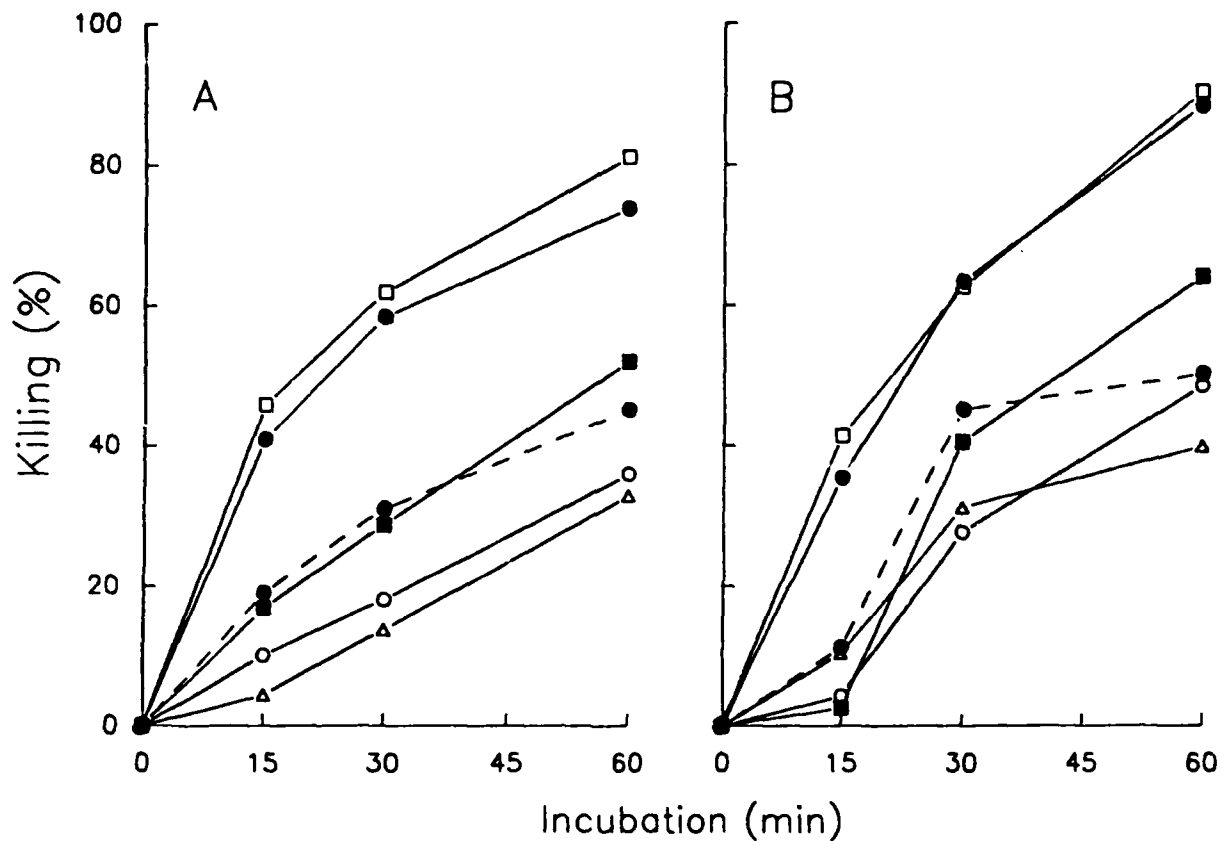


Figure 5. Effect of various concentrations of NSAIDs on the bactericidal activity of PMNs from thermally injured animals. PMNs harvested from injured animals at one day postburn were preincubated for 15 min at room temperature with piroxicam (A) or indomethacin (B) at the following concentrations before addition of the assay: 25 μM (○), 50 μM (■), 100 μM (●). The dashed line shows 100 μM piroxicam or indomethacin added to the PMNs without preincubation before assay. Untreated PMNs from injured animals (Δ) and normal animals (\square) were also assayed. Data are from a representative experiment run in duplicate.

cells was measured in the presence of increasing concentrations of purified PGE₁ or PGE₂. Addition of PGE₁ (source, Advanced Magnetics) to the assays inhibited the bactericidal activity of PMNs from injured animals that had been treated with piroxicam in vitro (Fig. 6) or in vivo (Fig. 7). At a concentration of 5 μM PGE₁, bactericidal activity was reduced to the level observed before treatment of the PMNs with piroxicam. In contrast to the results obtained with PGE₁, concentrations of 1 or 5 μM PGE₂ (source, Advanced Magnetics) had no effect on the bactericidal activity of PMNs from injured animals treated with piroxicam in vitro or in vivo (data not shown). The same PGE₂ preparation also did not inhibit the bactericidal activity of PMNs from normal animals, whereas dose-dependent inhibition of the bactericidal activity of normal PMNs was observed with PGE₁ (Fig. 8). Similar results were obtained with a second preparation of purified PGE₂ from a different source (i.e., Upjohn Diagnostics; data not shown). These results supported involvement of PGE₁ but not PGE₂ in the bactericidal defect of PMNs in the injured animals.

Effect of cAMP and theophylline on the bactericidal activity of PMNs. PGE₁ has been shown to inhibit various functions of PMNs by raising intracellular cAMP [16-18,20]. If this mechanism was responsible for the observed inhibitory effects of PGE₁ on PMN bactericidal activity, then similar effects should be achieved with other agents that increase cAMP and with cAMP itself. To test this hypothesis, PMNs from injured animals were treated with piroxicam in vitro or in vivo, and the bactericidal activity of these cells was measured in the presence of cAMP, dibutyryl cAMP, or theophylline. Like PGE₁, cAMP, dibutyryl cAMP, and theophylline markedly reduced the bactericidal activity of PMNs from injured animals treated with piroxicam in vitro or in vivo (Fig. 9). These agents also decreased the bactericidal activity of normal PMNs (Fig. 10). cAMP and dibutyryl cAMP

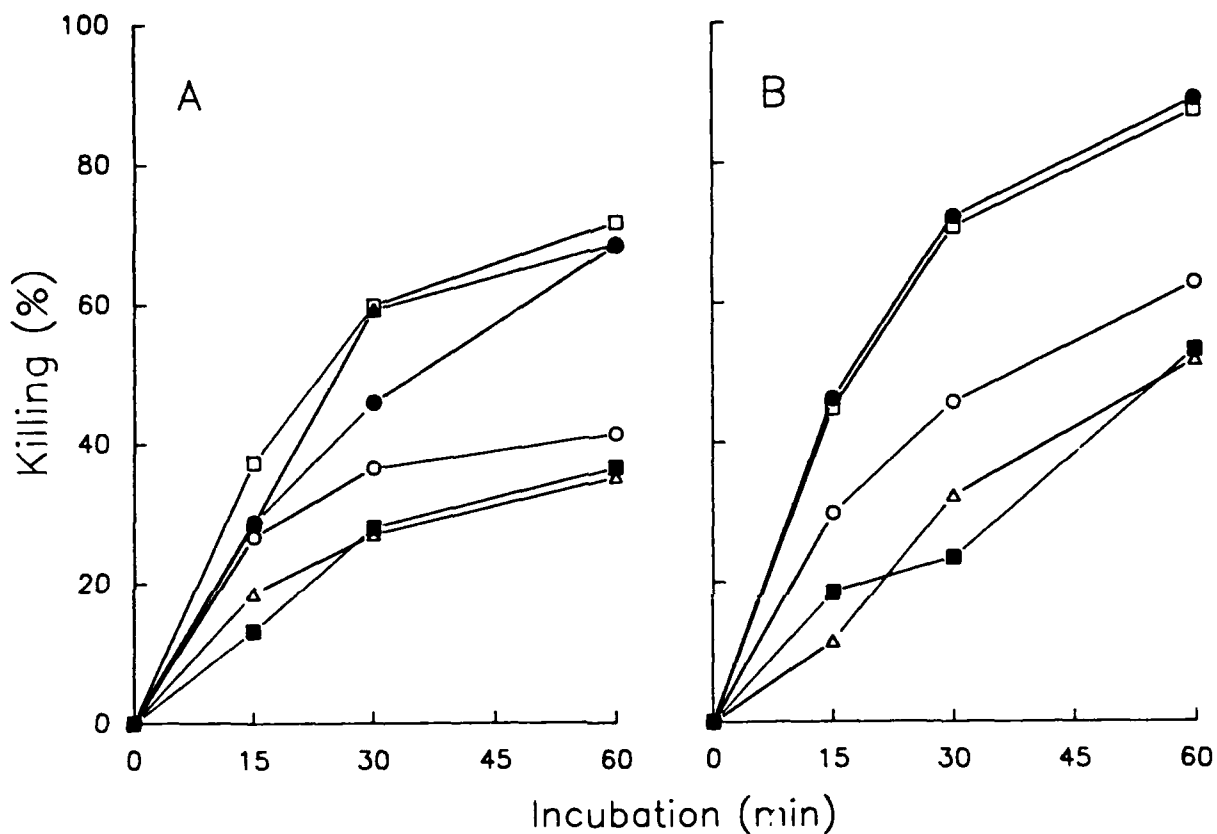


Figure 6. Effect of PGE₁ on the bactericidal activity of PMNs from thermally injured animals treated in vitro with piroxicam. PMNs harvested from injured animals at one (A) or two (B) days postburn were preincubated for 15 min at room temperature with 100 μM piroxicam or were untreated. PMNs treated with piroxicam were assayed together with 0.5 μM PGE₁ (▲), 1 μM PGE₁ (○), 5 μM PGE₁ (■), or buffer (●). Untreated PMNs from injured animals (△) and normal animals (□) were assayed in the absence of PGE₁. Data are from a representative experiment run in duplicate.

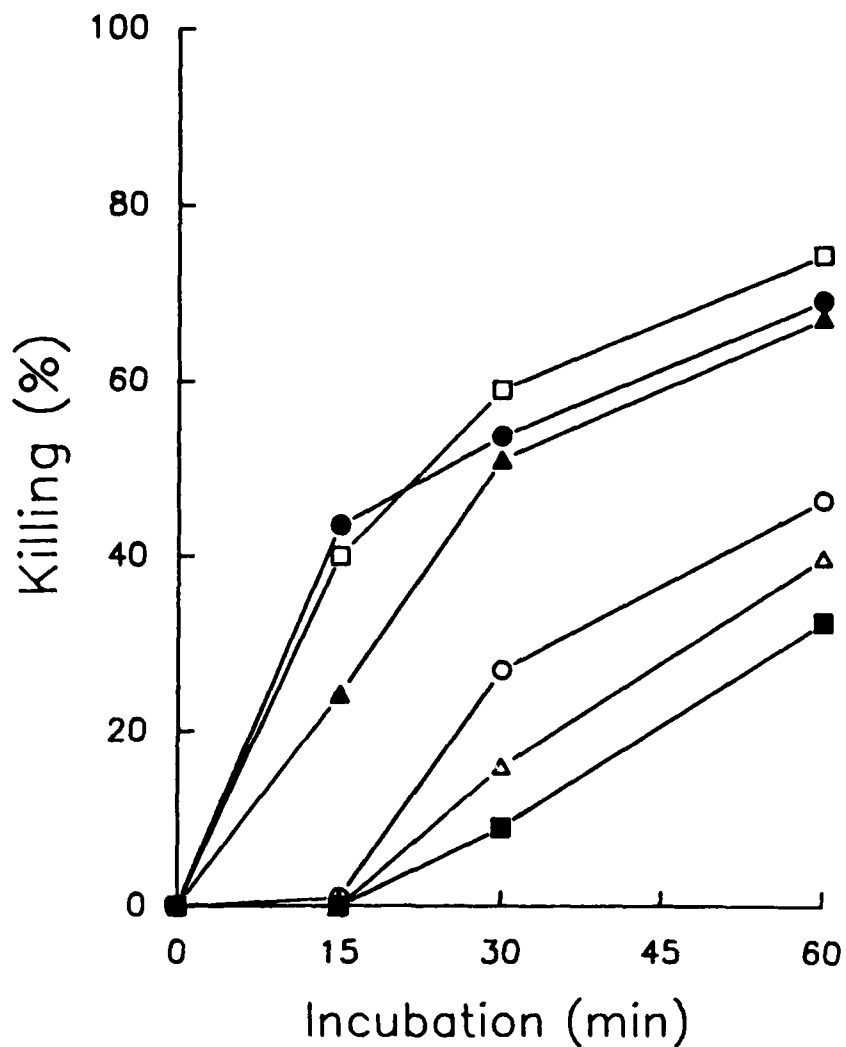


Figure 7. Effect of PGE₁ on the bactericidal activity of PMNs from thermally injured animals treated in vivo with piroxicam. PMNs were harvested at one day postburn from injured animals treated parenterally with piroxicam and from untreated animals. PMNs from animals treated with piroxicam were assayed together with 0.5 μM PGE₁ (▲), 1 μM PGE₁ (○), 5 μM PGE₁ (■), or buffer (●). PMNs from untreated injured animals (△) and normal animals (□) were assayed in the absence of PGE₁. Data are from a representative experiment run in duplicate.

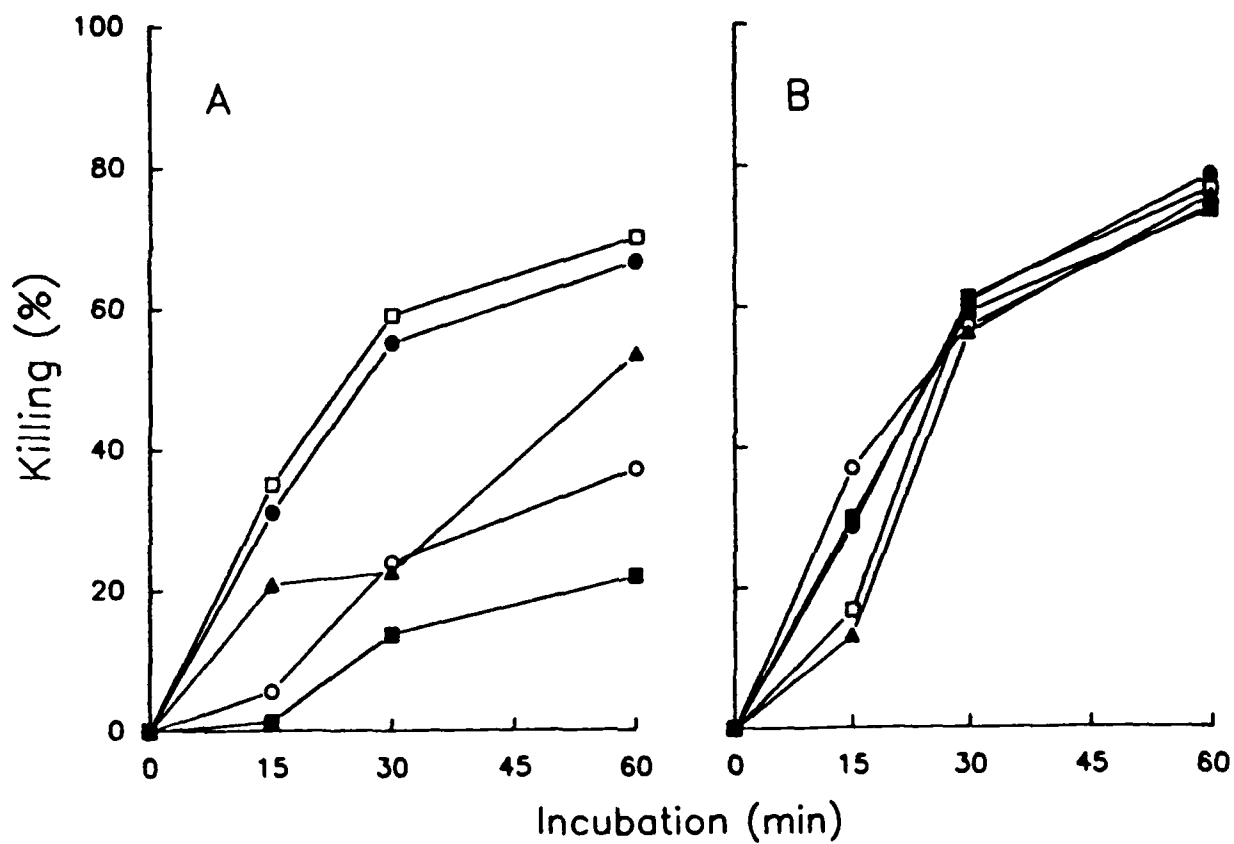


Figure 8. Effect of PGE₁ and PGE₂ on the bactericidal activity of PMNs from normal animals. Normal PMNs were assayed together with PGE₁ (A) or PGE₂ (B) at the following concentrations: 0.05 μM (●), 0.5 μM (▲), 1 μM (○), 5 μM (■). Normal PMNs were also assayed in the absence of PGE₁ or PGE₂ (□). Data are from a representative experiment run in duplicate.

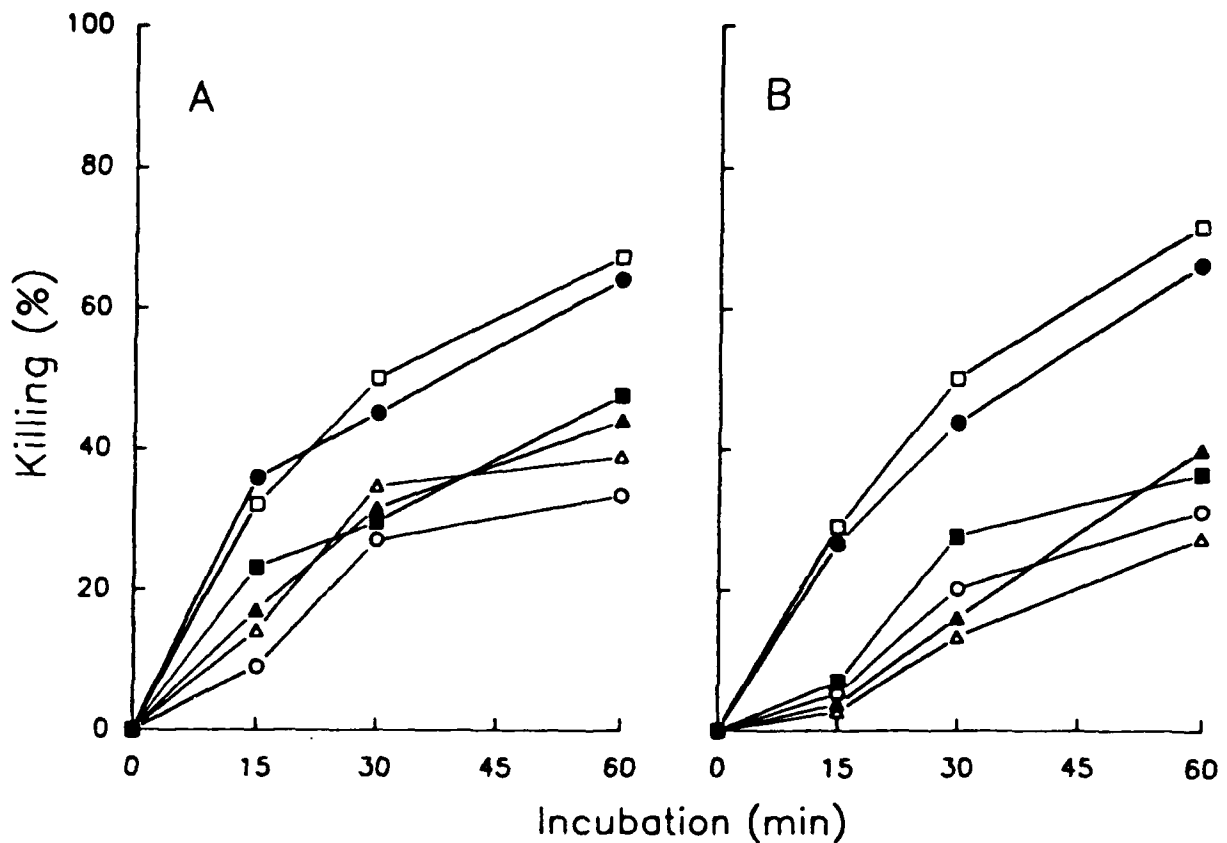


Figure 9. Effect of cAMP and theophylline on the bactericidal activity of PMNs from thermally injured animals treated in vitro or in vivo with piroxicam. PMNs harvested from injured animals at one day postburn were treated in vitro (A) or in vivo (B) with piroxicam or were untreated. PMNs treated with piroxicam were assayed together with 10 mM cAMP (■), 10 mM dibutyryl cAMP (○), 1 mM theophylline (▲), or buffer (●). Untreated PMNs from injured animals (Δ) and normal animals (□) were assayed in the absence of cAMP and theophylline. Data are from a representative experiment run in duplicate.

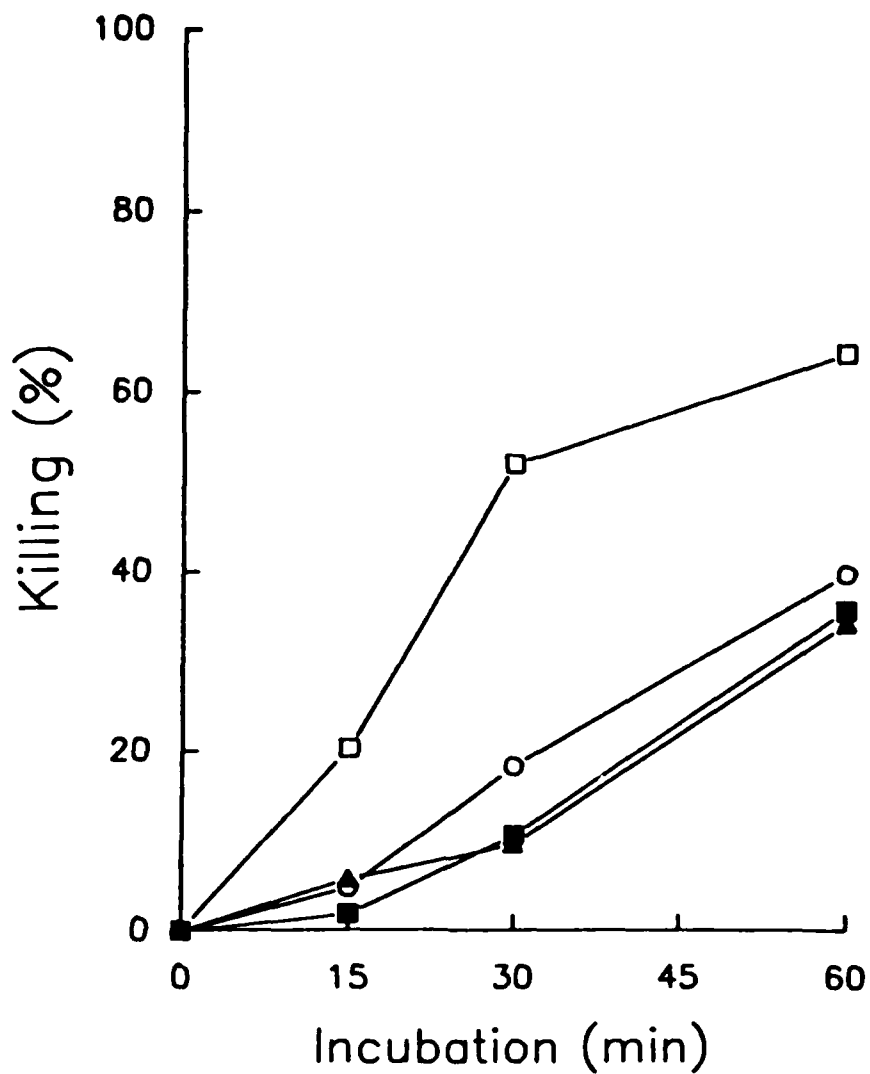


Figure 10. Effect of cAMP and theophylline on the bactericidal activity of PMNs from normal animals. Normal PMNs were assayed alone (□) and together with 10 mM cAMP (■), 10 mM dibutyryl cAMP (○), or 1 mM theophylline (▲). Data are from a representative experiment run in duplicate.

were both effective in reducing the bactericidal activity of PMNs. Dibutyl cAMP is more accessible for entry into cells than cAMP; however, both compounds appear to be capable of entering PMNs at the high concentrations used in our study. These results suggested that PGE₁ inhibited PMN bactericidal activity in the preceding experiments by raising intracellular cAMP.

cAMP accumulation in PMNs under various conditions. To further investigate the relationship between PMN bactericidal activity and intracellular cAMP, the cAMP content of PMNs from injured and normal animals was compared. In addition, the effect of treatment with NSAIDs and cAMP activators on cAMP content was determined under conditions identical to those used in the bactericidal assays. The cAMP content was approximately five-fold higher in PMNs from injured animals than in PMNs from normal animals (Table I). Treatment of PMNs from injured animals with NSAIDs in vitro or in vivo markedly reduced cAMP. When PMNs from injured animals were treated with piroxicam in vivo and then incubated with PGE₁, the cAMP content increased. Major increases in cAMP content were also observed upon treatment of normal PMNs with PGE₁ or theophylline, but not with PGE₂. These results suggested that NSAIDs and the cAMP activators modulated PMN bactericidal activity through effects on cAMP.

Relationship between cAMP accumulation and PGE₁ production by PMNs. To determine if the elevation of cAMP in PMNs from injured animals was associated with increased production of PGE₁ by these cells, spontaneous production of PGE₁ by PMNs from injured and normal animals was compared. The effect of NSAIDs on PGE₁ production by PMNs from injured animals was also determined. PMNs from injured animals produced approximately threefold more PGE₁ than PMNs from normal animals (Table II). Treatment of PMNs from injured animals with NSAIDs in vitro or in vivo reduced PGE₁ production to the level observed with normal PMNs. These results suggested that the

TABLE I
cAMP content of PMNs under various conditions^a

Source of PMNs	Treatment of PMNs		cAMP content (pmol/10 ⁷ cells)
	NSAID	cAMP activator	
Injured animals (day 1 postburn)	None	None	109.3 ± 10.1
	Indomethacin (in vitro)	None	44.8 ± 3.0
	Ibuprofen (in vitro)	None	45.0 ± 2.0
	Piroxicam (in vitro)	None	46.8 ± 0.8
	Piroxicam (in vivo)	None	29.3 ± 0.3
	Piroxicam (in vivo)	PGE ₁	92.0 ± 2.6
Normal animals	None	None	19.8 ± 1.1
	None	PGE ₁	97.0 ± 5.9
	None	PGE ₂	40.5 ± 2.9
	None	Theophylline	144.3 ± 5.4

^aPMNs were first preincubated for 15 min at room temperature with 100 μM NSAID or were treated with piroxicam in vivo as described in the *Methods* section. PGE₁ (5 μM), PGE₂ (5 μM), or theophylline (1 mM) were then added to the PMNs; under these conditions, the cells were incubated for 2 min at 37°C before boiling and sonication. Data are from a representative experiment. Data are mean ± SEM; n, 4.

TABLE II
Effect of NSAIDs on PGE₁ production by PMNs^a

Source of PMNs	Treatment of PMNs	PGE ₁ production (pg/10 ⁷ cells)
Injured animals (day 1 postburn)	None	19,032 ± 196
	Indomethacin (in vitro)	5,841 ± 304
	Ibuprofen (in vitro)	6,139 ± 359
	Piroxicam (in vitro)	6,114 ± 316
	Piroxicam (in vivo)	4,551 ± 433
Normal animals	None	6,490 ± 146

^aPMNs were first preincubated for 15 min at room temperature with 100 μM NSAID or were treated with piroxicam in vivo as described in the *Methods* section. PMNs were then incubated for 15 min at 37°C in the absence of exogenous PMN activators, and the amount of PGE₁ released into the supernatants was determined. Data are from a representative experiment. Data are mean ± SEM; n, 4.

increased production of PGE₁ by PMNs from injured animals contributed to the elevation of cAMP within these cells.

To further determine the relationship between cAMP content, PGE₁ production, and bactericidal activity, these parameters were measured in PMNs harvested from injured animals at one, two, four, and seven days postburn. Bactericidal activity was restored to normal during four to seven days postburn (Table III). cAMP content and PGE₁ production were reduced concomitantly during this period. These results provided additional evidence that the bactericidal defect of PMNs induced by thermal injury was related to elevation of cAMP and that PGE₁ contributed to this phenomenon.

Postburn changes in cAMP content, PGE₁ production, and bactericidal activity of peritoneal exudate PMNs. The relationship between cAMP content, PGE₁ production, and bactericidal activity was also investigated using PMNs harvested from peritoneal exudates. The trend of results using PMNs from this source (Table IV) was similar to that obtained using peripheral PMNs (Table III), except the extent of elevation of cAMP and PGE₁ production by peritoneal exudate PMNs was less than that observed with peripheral PMNs (compare Tables III and IV). This difference in results may be related to the greater purity of the peritoneal exudate PMN preparations (>95% pure) as compared with the peripheral PMN preparations (≥85% pure). Some of the PGE produced by the peripheral PMN preparations may be derived from contaminating mononuclear cells and/or possibly also platelets, which have a more active cyclooxygenase system. The additional PGE produced by these cells may boost cAMP levels in the PMNs.

Discussion and Conclusions

Previous studies have suggested that cAMP plays an important role in the negative feedback control of PMN effector functions [16-18,20,29-32].

TABLE III

Natural changes in cAMP content, PGE₁ production, and bactericidal activity of PMNs during the first week postburn^a

Source of PMNs	Days postburn	cAMP content (pmol/10 ⁷ cells)	PGE ₁ production (pg/10 ⁷ cells)	Killing (%)		
				15 min	30 min	60 min
Injured animals	1	114.0 ± 6.5	20,588 ± 293	0	8	40
	2	94.5 ± 0.9	18,416 ± 772	1	19	31
	4	70.3 ± 5.8	16,474 ± 745	15	32	69
	7	46.1 ± 5.7	12,491 ± 397	20	53	64
Normal animals		19.6 ± 1.2	8,288 ± 498	26	53	65

^aBactericidal activity was assayed in duplicate. Other data are mean ± SEM; n, 4.

TABLE IV
 Postburn changes in cAMP content, PGE₁ production, and bactericidal
 activity of peritoneal exudate PMNs^a

Source of PMNs	Days postburn	cAMP content (pmol/10 ⁷ cells)	PGE ₁ production (pg/10 ⁷ cells)	Killing (%)		
				15 min	30 min	60 min
Injured animals	1	79.8 ± 2.7	11,956 ± 301	15 ± 6	23 ± 2	34 ± 4
	2	60.0 ± 6.5	10,836 ± 684	16 ± 5	30 ± 5	40 ± 4
	4	41.8 ± 2.5	9,974 ± 718	23 ± 4	31 ± 5	47 ± 5
	7	20.3 ± 2.4	7,973 ± 355	31 ± 5	44 ± 4	58 ± 2
Normal animals		10.3 ± 0.5	5,747 ± 254	36 ± 2	56 ± 2	71 ± 1

^aData are mean ± SEM; n, 3 (bactericidal activity) or n, 4 (cAMP content and PGE₁ production).

These studies have shown that exposure of normal PMNs in vitro to high concentrations of cAMP or to agents that markedly elevate intracellular cAMP diminishes chemotaxis, phagocytosis, degranulation, oxidative metabolism, and iodination. The present investigation is the first to demonstrate the natural occurrence of this mechanism in vivo in the heightened inflammatory state associated with thermal injury. Using a guinea pig model of thermal injury, we demonstrated that peripheral and peritoneal exudate PMNs from injured animals had a marked elevation of cAMP as compared with similar PMN preparations from normal animals. This elevation of cAMP was associated with defective bactericidal activity against *P. aeruginosa*. The bactericidal defect was corrected by treating peripheral PMNs from the injured animals with NSAIDs in vitro or in vivo, with concomitant reduction in the cAMP content of these cells. When cAMP was reelevated by treating the PMNs with PGE₁, theophylline, or with cAMP itself, bactericidal activity was again reduced to an abnormally low level. Thus, by artificially raising and lowering the cAMP content of PMNs from the injured animals, we were able to inversely modulate bactericidal activity.

Natural changes in cAMP content of PMNs from the injured animals and associative changes in bactericidal activity during the first week postburn were also studied. cAMP in these cells was maximally increased and bactericidal activity was maximally reduced at one day postburn. By four days postburn, the cAMP content had declined and, by seven days postburn, a major reduction in cAMP was observed. Bactericidal activity gradually increased during this time period and was restored to an approximately normal level by seven days postburn. The extent of the reduction in the cAMP content at seven days postburn was equivalent to that observed when PMNs obtained early after injury were treated with NSAIDs in vitro. These observations taken together with those described above provide strong support

for the hypothesis that the bactericidal defect of PMNs induced by thermal injury is related to a marked elevation of cAMP.

Our study also investigated the mechanisms responsible for the increase in cAMP in PMNs from the injured animals. Several pieces of evidence were obtained suggesting that PGE₁, the major E type prostaglandin produced by activated PMNs [33], plays a significant role in this phenomenon. First, peripheral and peritoneal exudate PMNs from the injured animals produced considerably more PGE₁ than similar PMN preparations from normal animals when incubated in vitro in the absence of exogenous PMN activators. Secondly, spontaneous PGE₁ production and the cAMP content of these cells were reduced concomitantly as bactericidal activity was restored to normal during four to seven days postburn and also under conditions in which peripheral PMNs obtained early after injury were treated in vitro or in vivo with NSAIDs. Thirdly, a concentration of 100 μM NSAID was required to normalize the bactericidal activity of these cells in vitro; this drug concentration was also required to effectively reduce the cAMP content and PGE₁ production by these cells (data not shown).

PGE₂ was not found to play a major role in the elevation of cAMP in PMNs from the injured animals. Purified PGE₂ from two different sources had no effect on the bactericidal activity of normal PMNs or PMNs from injured animals that had been treated in vitro or in vivo with piroxicam; bactericidal activity of PGE₂-treated cells was equal to that of normal PMNs. In addition, incubation of normal PMNs with purified PGE₂ at a concentration of 5 μM caused only a minimal increase in cAMP. Thus, on a molar basis, PGE₂ was considerably less efficient than PGE₁ in raising the cAMP content of PMNs, and this presumably explains why it had no effect on bactericidal activity.

Ishitoya and Takenawa have recently demonstrated that PGE₁ acts synergistically with substances that activate PMNs to increase and maintain intracellular cAMP at very high levels [34]. This mechanism may be involved in the elevation of cAMP observed in our study. A number of mediators produced in response to thermal injury have the potential to increase cAMP in PMNs. Our previous studies have shown that complement cleavage fragments are present in circulation in injured animals early after injury [14], and Moore et al. have suggested that these fragments contribute to the activation of PMNs in injured patients [12]. Substances released from damaged tissue and bacterial products formed as a result of bacterial colonization of the burn wound may also be involved in PMN activation and the associated increase in cAMP. Catecholamines and glucocorticoids are thought to be responsible for the hypermetabolic response to thermal injury [35,36], and these substances are known to have profound effects on cAMP accumulation. Like PGE₁, catecholamines increase cAMP in PMNs by acting as adenylyl cyclase agonists [37], whereas glucocorticoids potentiate the effects of these agents [20].

Our study did not prove that NSAIDs lowered the cAMP content of PMNs from the injured animals entirely through inhibition of PGE₁ production. Other mechanisms may also be involved. Concentrations of NSAIDs as high as those used in our in vitro studies have been shown to inhibit the activation of normal PMNs [38-40]. It has been proposed that NSAIDs interfere with the triggering of cellular processes by blocking the interaction between the G protein and the receptor for the ligand [40]. However, this mechanism would not explain our finding that NSAIDs corrected the bactericidal defect of PMNs from the injured animals when employed in vitro, since activation of these cells had presumably already occurred in vivo. There is little known about the effects of NSAIDs on PMNs after they have under-

gone activation; however, our results suggest that these drugs modulate cAMP during this phase.

One of the most significant observations of our study is that the bactericidal defect of PMNs induced by thermal injury is reversible. It has been previously postulated that the depression in PMN effector functions associated with thermal injury reflects a state of irreversible cell exhaustion following maximal stimulation. Our study indicates that this is not correct with respect to bactericidal activity. However, it is possible that other effector functions of these cells, such as chemotaxis, may be irreversibly altered by thermal injury, even though cAMP accumulation in these cells can be effectively reduced by pharmacologic means. The reduction in cAMP would facilitate reversal of microtubular disassembly [41]. However, the depression in chemotaxis is also associated with depletion of PMN specific granules, increased expression of the complement receptors, CR1 and CR3, and internalization of C5a receptors [9,10,12]. These events may not be reversible.

The elevation of cAMP in PMNs produced in response to injury and the associated depression in effector functions may have been designed teleologically to protect the host from the harmful consequences of PMN activation. At present, it is not known whether therapy with NSAIDs by increasing bactericidal activity would increase resistance against infection, or whether this therapy would allow for continued PMN activation with associated adverse effects on the host. Our observation that piroxicam was capable of reducing the cAMP content of PMNs and normalizing bactericidal activity in vivo despite ongoing inflammation suggests that this drug may be capable of beneficially modulating PMN activation in an inflammatory environment. The clinical application of these findings is dependent on understanding the mechanisms responsible for and the reversibility of other PMN alterations induced by severe injury.

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