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Human Monocyte Killing of *Staphylococcus aureus*: Modulation by Agonists of Cyclic Adenosine 3',5'-Monophosphate and Cyclic Guanosine 3',5'-Monophosphate

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This study was designed to test whether cyclic nucleotides play a role in the regulation of bacterial killing by human monocytes. Agents were tested for their ability to activate monocyte adenylate or guanylate cyclase in cell-free preparations, to increase cyclic adenosine 3',5'-monophosphate (cAMP) or cyclic guanosine 3',5'-monophosphate (cGMP) in intact human monocytes, and to modulate monocyte-induced killing of *Staphylococcus aureus* in vitro. Prostaglandin E_1 and cholera toxin activated monocyte adenylate cyclase and inhibited monocyte killing of *S. aureus*. An adenylate cyclase inhibitor, RMI 12330A, reversed the prostaglandin E_1-mediated inhibition of bacterial killing, thus implicating cAMP as the intracellular mediator of this inhibition. In contrast, monocyte cGMP levels were increased 5- and 17-fold by 5-hydroxytryptamine and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, respectively, but neither agent was effective in modulating monocyte bactericidal activity. Thus, modulation of bactericidal activity in human monocytes did not conform to the yin/yang theory of opposing actions by cAMP and cGMP, for although monocyte-mediated killing of *S. aureus* was inhibited by cAMP agonists, it was not enhanced by cGMP agonists.

Monocytes and macrophages play important roles in many aspects of the immune response. They interact with T and B lymphocytes to initiate cellular and humoral immune responses (33, 34, 39, 45), they sequester dying erythrocytes (11), and they secrete factors necessary for leukocyte growth and differentiation (5, 21). In addition, monocytes and macrophages are directly cytotoxic for tumor cells (32), antibody-coated erythrocytes (38), viruses (19), parasites (8), and bacteria (18).

Cyclic adenosine 3',5'-monophosphate (cAMP) modulation has been demonstrated in several models of macrophage function. In animal models, increases in cAMP are associated with inhibition of plasminogen activator and heme oxygenase synthesis (11, 46), chemotaxis (2), and tumoricidal activity (7, 37). On the other hand, exogenous cAMP restores normal phagocytosis in an insulin-inhibited macrophage cell line (26). Cyclic guanosine 3',5'-monophosphate (cGMP) modulation of macrophage function is not yet understood. Agents which increase cGMP enhance chemotaxis (35), but the effect of cGMP agonists on bacterial and tumor cell killing remain to be elucidated.

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Although peripheral blood monocytes have long been recognized as the precursor of the tissue macrophage and are now recognized as active effector cells in the peripheral blood (18, 32, 38), their cyclic nucleotide metabolism is not well characterized. We have studied the synthesis of both cAMP and cGMP in human monocytes and have measured the effect of increases in each of these nucleotides on the ability of monocytes to kill *Staphylococcus aureus* in vitro.

MATERIALS AND METHODS

Monocyte purification. Mononuclear cells were purified from peripheral blood by Ficoll-Hypaque gradient centrifugation(3). Monocytes were purified further by adherence to tissue culture plates in Hanks balanced salt solution containing 12% human AB serum (20). After a 90-min incubation, nonadherent cells were removed by washing vigorously with Hanks balanced salt solution, leaving monolayers containing >95% monocytes.

Quantification of intracellular cyclic nucleotides. After removal of nonadherent cells and platelets, each monocyte monolayer was replenished with Hanks balanced salt solution for the remainder of the study. Experimental drugs were dissolved in Hanks balanced salt solution. Reactions were terminated by the addition of 30% trichloroacetic acid to each layer, resulting in a final concentration of 10% trichloroacetic

acid. The monocytes were then removed from the plates with a rubber policeman. The contents of three layers (approximately 7×10^6 monocytes) were pooled for purification of cyclic nucleotides by ion-exchange chromatography and measurement by radioimmunoassay by the method of Steiner et al. (41, 42). cGMP samples were succinylated before assay to increase the sensitivity of the assay (10). Recovery was monitored by subjecting [^{3}H]cGMP and [^{14}C]cAMP to the same extraction and chromatography procedures. Recovery of cAMP was 85 to 95%, and recovery of cGMP was 75 to 80%. The cross-reactivity of our cGMP antiserum with guanosine 5'-triphosphate, guanosine 5'-diphosphate, and GMP was less than 1:100,000. The cross-reactivity of cGMP antiserum with cAMP was less than 1:20,000. cAMP antiserum was obtained commercially. Its cross-reactivity was rechecked by us and found to be less than 1:20,000 for cGMP and less than 1:100,000 for adenosine 5'-triphosphate, adenosine 5'-diphosphate, and AMP. The cell number was determined by deoxyribonucleic acid assay (4).

Adenylate and guanylate cyclase assays. Hanks balanced salt solution was poured off the monolayers and replaced with 5 mM tris(hydroxymethyl)aminomethane (Tris)–0.25 M sucrose, pH 7.4. Monocytes were then removed from the plates with a rubber policeman. Approximately 20×10^6 monocytes were suspended in 1 ml of Tris-sucrose and sonicated for 2 min at a 30% maximum setting with a Sonic Dismembrator (Artek Systems Corp., Farmingdale, N.Y.). This technique disrupted >90% of the cells. Adenylate cyclase was assayed by a modification of the method of Remold-O'Donnell (30, 31). Samples of the enzyme preparation were incubated for 30 min at 37°C in a total volume of 0.1 ml containing 1 mM adenosine 5'-triphosphate, 2 mM $MgCl_2$, 10 mM theophylline, 0.1 mM dithiothreitol, 0.4% albumin, 3 mM phosphocreatine, 3 U of creatine kinase, and 25 mM Tris-hydrochloride, pH 7.7. The reaction was stopped by the addition of 0.9 ml of cold 0.1 M sodium acetate, pH 4, followed by heating for 3 min at 90°C. Product formation was quantified by radioimmunoassay as outlined above. cAMP generation was linear over a 40-min time course and showed linear protein dependence between 20 and 100 μg of protein as determined by the Lowry assay (25). Guanylate cyclase was assayed by a modification of the method of Kimura and Murad (17). Samples of the enzyme preparation were incubated for 15 min at 37°C in a total volume of 0.1 ml containing 1 mM guanosine 5'-triphosphate, 3 mM $MnCl_2$, 10 mM theophylline, 3 mM phosphocreatine, 3 U of creatine kinase, and 50 mM Tris-hydrochloride, pH 7.4. The reaction was terminated by the addition of 0.9 ml of cold 0.1 M sodium acetate, pH 4, followed by heating for 3 min at 90°C. Product formation was quantified by radioimmunoassay. This enzyme assay was linearly dependent on time between 2 and 20 min of incubation and on protein concentration between 10 and 50 μg.

Phagocytosis assay. Monocyte phagocytic capacity was measured by the method of Stossel et al. (43). Mononuclear cell preparations containing 10×10^6 monocytes ± 0.01 mM prostaglandin E_1 (PGE_1) were incubated in the presence of Oil Red O which had been opsonized with human AB serum. After 30 min, the cells were centrifuged and uningested particles were removed. Cells were dissolved in dioxane, and the uptake of Oil Red O was quantified spectrophotometrically.

Lysosomal enzyme release. Mononuclear cell preparations containing 10×10^6 monocytes ± 0.01 mM PGE_1 were incubated in the presence of opsonized zymosan (49) for 30 min. The cells were pelleted, and the incubation medium was removed for assay of β-glucuronidase and lactate dehydrogenase. The release of these enzymes during phagocytosis was compared with their release by resting cells. β-Glucuronidase was measured spectrophotometrically, using phenophthalein glucuronide as the substrate (9). Lactate dehydrogenase was also measured spectrophotometrically, using pyruvate and reduced nicotinamide adenine dinucleotide as substrates (16).

Bactericidal assay. The ability of human monocytes to kill bacteria was measured as described by King et al. (18). Mononuclear cells containing 2.5×10^6 monocytes were incubated with 12.5×10^6 *S. aureus* organisms in the presence of human AB serum. After 60 min, samples of the reaction were lysed in distilled water, and a sample was cultured for 48 h to determine the number of live bacteria by colony counts. The number of colonies at 60 min was subtracted from the number at zero time to calculate the number of bacteria killed.

Materials. PGE_1 was a gift from The Upjohn Co., Kalamazoo, Mich. RMI 12330A was provided by Merrell-National Laboratories, Cincinnati, Ohio. Cholera toxin was purchased from Schwarz/Mann, Orangeburg, N.Y.; 5-hydroxytryptamine (5-HT), zymosan, and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) were purchased from Sigma Chemical Co., St. Louis, Mo.

RESULTS

cAMP was increased in human monocytes exposed to PGE_1. Figure 1 shows the concentration dependence of the intracellular cAMP levels. PGE_1 at a concentration of 10 μM caused an eightfold increase in monocyte cAMP levels, from 0.5 ± 0.1 to 4.2 ± 0.4 pmol/10^6 cells, whereas 1 μM PGE_1 caused a fourfold increase. No change was effected by 0.1 μM PGE_1. The time course of these PGE_1-induced changes is shown in Fig. 2. The highest level of cAMP, 4.3 ± 0.2 pmol/10^6 monocytes, was seen 5 min after PGE_1 administration. cAMP levels declined thereafter, but remained significantly elevated (2.8 ± 0.6 pmol/10^6 monocytes).

PGE_1 added to homogenates prepared from human monocytes caused activation of adenylate cyclase. As shown in Table 1, 100 μM PGE_1 increased adenylate cyclase activity nearly sixfold. Although not shown in Table 1, this enzyme response was concentration dependent; adenylate cyclase activity was 45 and 25 pmol of cAMP per mg of protein per min in the presence of 10

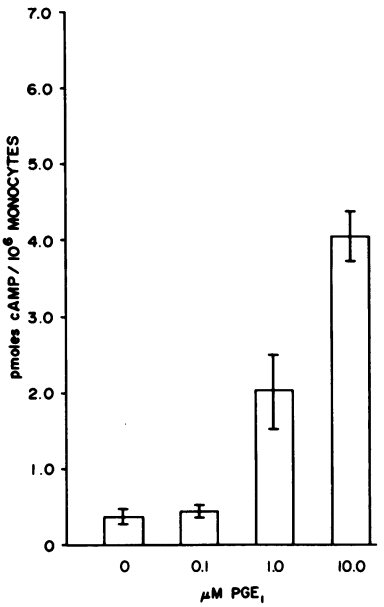

FIG. 1. *Effect of PGE_1 on cAMP levels in human monocytes. Monocytes were incubated with the indicated concentrations of PGE_1 for 10 min, the reaction was stopped by the addition of trichloroacetic acid, and cyclic nucleotides were extracted and purified by column chromatography for quantification by radioimmunoassay. Values are the mean ± the standard error of the mean for three experiments. The statistical evaluation indicates no significance, $P < 0.05$, and $P < 0.001$ for 0.1, 1.0, and 10 μM PGE_1, respectively.*

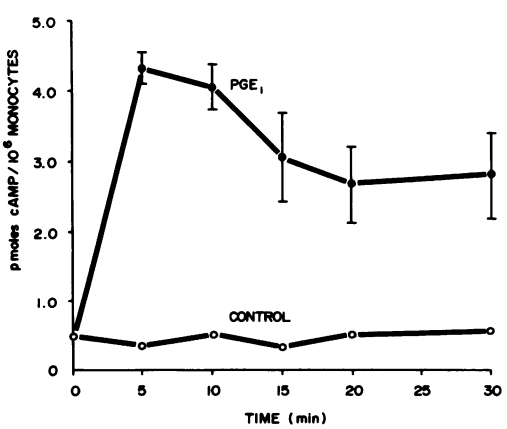

FIG. 2. *Time course of PGE_1-induced increase in monocyte cAMP levels. Monocytes were incubated with or without 10 μM PGE_1. The reaction was stopped at the indicated times by the addition of trichloroacetic acid. Cyclic nucleotides were extracted, purified by column chromatography, and quantified by radioimmunoassay. Values are the average ± the standard error of the mean for triplicate determinations. All PGE_1 values are statistically different from the control with $P < 0.005$.*

TABLE 1. *Monocyte adenylate cyclase*[a]

Medium	RMI 12330A	pmol of cAMP per mg per min
Basal	−	9 ± 1
NaF	−	32 ± 7[b]
PGE_1	−	50 ± 7[b]
Cholera toxin	−	17 ± 1[b]
NaF	+	10 ± 2[c]
PGE_1	+	14 ± 3[c]
Cholera toxin	+	7 ± 1[c]

[a] Monocytes were sonicated in 5 mM Tris–0.25 M sucrose, pH 7.4, and assayed for adenylate cyclase activity. NaF (10 mM) or PGE_1 (0.1 mM) ± 1 mM RMI 12330A was added immediately before the addition of assay medium. Cholera toxin (1 μg/ml) ± RMI 12330A was incubated with monocytes for 1 h before cells were disrupted. Values are expressed as the mean ± the standard error of the mean for three experiments.

[b] Statistically different from control with $P < 0.01$.

[c] Statistically different from NaF, PGE_1, or cholera toxin alone with $P < 0.01$.

and 1 μM PGE_1, respectively. Table 1 also demonstrates the response of monocyte adenylate cyclase to cholera toxin. Incubation of cholera toxin with intact monocytes before disruption of the cells caused a twofold increase in adenylate cyclase activity. Sodium fluoride (NaF) stimulation of monocyte adenylate cyclase is included in Table 1 for a reference. NaF, like PGE_1, can be added directly to broken cell preparations. NaF stimulates adenylate cyclase by a mechanism independent of membrane receptors. Because PGE_1, cholera toxin, and NaF differ in their requirement for plasma membrane integrity, the degree of activation effected in homogenates may not be strictly comparable to the effectiveness of these same agents in modulating cAMP in intact monocytes. In fact, NaF does not increase cAMP levels in intact cells (28). However, activation of monocyte adenylate cyclase by all three agents was prevented by agent RMI 12330A. PGE_1-induced increases in intracellular cAMP were also prevented by RMI 12330A (data not shown).

cGMP was increased in human monocytes by sodium azide (NaN_3), 5-HT, and MNNG, as shown in Table 2. In the presence of the cyclic nucleotide phosphodiesterase inhibitor methylisobutylxanthine, monocyte cGMP levels were increased 3-fold by NaN_3, 5-fold by 5-HT, and 17-fold by MNNG. However, only NaN_3 and MNNG were able to activate guanylate cyclase in disrupted cell preparations. Having demonstrated modulation of both adenylate and guanylate cyclases, we looked at the effect of increasing intracellular cAMP and cGMP levels on monocyte bactericidal activity. Both cholera toxin and PGE_1 inhibited monocyte-induced

TABLE 2. *Monocyte cGMP metabolism*[a]

Medium	Guanylate cyclase (pmol of cGMP per mg per min)	Cellular cGMP (pmol of cGMP per 10^6 cells)
Basal	41 ± 6	0.060 ± 0.016
NaN_3	128 ± 23[b]	0.207 ± 0.038[b]
5-HT	73 ± 20	0.342 ± 0.054[b]
MNNG	138 ± 33[b]	1.022 ± 0.300[b]

[a] Guanylate cyclase was assayed in homogenates of human monocytes. NaN_3 (10 mM), 5-HT (0.1 mM), or MNNG (0.1 mM) was preincubated with the enzyme for 20 min at 4°C. Values are the average ± the standard error of the mean for five experiments, except MNNG for which $n = 3$. Intracellular cGMP was measured in purified monocyte layers. Cells were preincubated for 5 min in the presence of 1 mM methyl isobutylxanthine. Agents were added in the same concentration as used in the enzyme assay, and the monocytes were incubated for an additional 5 min. Values are expressed as the average ± the standard error of the mean for three experiments.

[b] Statistically different from basal with $P < 0.01$.

killing of *S. aureus*. Cholera toxin inhibited killing by 30% when incubated with monocytes at a concentration of 10 ng/ml and by 50% at 100 ng/ml (Fig. 3). PGE_1 (10 μM) also inhibited monocyte bactericidal activity, but this inhibition could be prevented by the adenylate cyclase inhibitor RMI 12330A (Fig. 4). RMI 12330A alone had no effect on monocyte bactericidal activity (data not shown). Thus, inhibition of monocyte bactericidal activity by PGE_1 appears to depend on the activation of adenylate cyclase. The cAMP-mediated inhibition of bacterial killing was investigated further by examining the effect of PGE_1 on phagocytosis and lysosomal enzyme release. Table 3 shows the effect of PGE_1 on phagocytosis of lipopolysaccharide particles containing Oil Red O. The amount of Oil Red O ingested was not affected by preincubation of the monocytes with 0.01 mM PGE_1. However, preincubation of monocytes with 0.01 mM PGE_1 did decrease lysosomal enzyme release during phagocytosis of zymosan particles (Table 3). The mean decrease in the release of the lysosomal enzyme β-glucuronidase was 37% ($P < 0.025$ by a paired-t analysis). The release of the cytosolic enzyme lactate dehydrogenase was unchanged during phagocytosis.

In contrast to cholera toxin and PGE_1, neither 5-HT nor MNNG had any effect on the ability of monocytes to kill *S. aureus* (Table 4). The ability of 5-HT to modulate monocyte bactericidal activity was tested at 1, 0.1, and 0.01 mM. Although 0.1 mM 5-HT increased cGMP levels fivefold, it neither enhanced nor inhibited bactericidal activity. MNNG is a more potent cGMP agonist, but even 0.1 mM MNNG, which increased cGMP levels 17-fold, had no effect on bactericidal activity. In other experiments, the monocyte/bacteria ratio was adjusted to 1:3 and 1:10, but, again, neither 5-HT nor MNNG had any effect on bacterial killing. Because cGMP

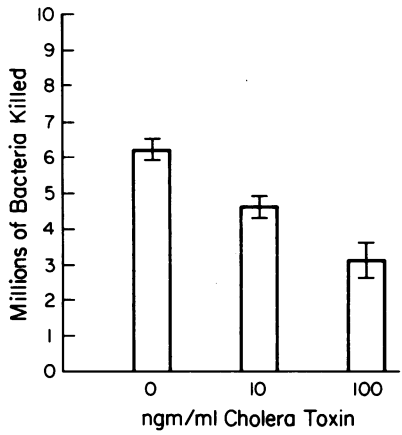

FIG. 3. *Effect of cholera toxin on monocyte bactericidal activity. Mononuclear cells were preincubated for 30 min with the indicated amounts of cholera toxin. A total of 2.5 × 10^6 monocytes were then incubated with 12.5 × 10^6 S. aureus organisms for 60 min. Samples were withdrawn, cells were lysed, and the numbers of viable bacteria were determined by colony counts. The decrease in killing is significant with P < 0.005 and P < 0.001 for 10 and 100 ng of cholera toxin, respectively.*

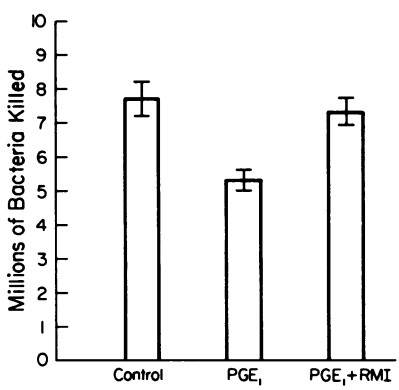

FIG. 4. *Effect of PGE_1 on monocyte bactericidal activity. A total of 2.5 × 10^6 monocytes were incubated with 12.5 × 10^6 S. aureus organisms and 10 μM PGE_1 ± 100 μM RMI 12330A. After 60 min, samples were withdrawn, cells were lysed, and the numbers of viable bacteria were determined by colony counts in 48-h cultures. Values are the average ± the standard error of the mean for three experiments. The decrease in killing is significantly different from the control in the presence of PGE_1 ($P < 0.01$), but not in the presence of PGE_1 + RMI 12330A. The increase in killing for PGE_1 + RMI 12330A compared with PGE_1 alone is statistically significant with $P < 0.01$.*

TABLE 3. *Effect of PGE_1 on phagocytosis and lysosomal enzyme release by monocytes*[a]

Cells	PGE_1	μg of paraffin oil per 10^7 cells	U of β-glucuronidase per 16 h per 10^7 cells	U of lactate dehydrogenase per min per 10^7 cells
Resting	−	0	65	62
Phagocytic	−	610	153[b]	58
Phagocytic	+	610	120[b,c]	64

[a] Monocytes were incubated in the presence of human AB serum and paraffin Oil Red O emulsified with bacterial lipopolysaccharide. Cells and ingested paraffin oil were dissolved in dioxane. The amount of paraffin oil ingested was determined spectrophotometrically. β-Glucuronidase and lactate dehydrogenase were measured in media after monocytes were incubated with opsonized zymosan. Units of β-glucuronidase activity are measured as nanomoles of phenolphthalein released from phenolphtholein glucuronide. Less than 10% of the total β-glucuronidase was released from resting cells. Units of lactate dehydrogenase are defined as nanomoles of reduced nicotinamide adenine dinucleotide oxidized. Less than 5% of the total lactate dehydrogenase was released under any of the conditions assayed.

[b] Statistically different from resting with $P < 0.025$, using paired-t analysis of four experiments.

[c] Statistically different from phagocytic without PGE_1 with $P < 0.025$, using paired-t analysis of four experiments.

increases in other tissues are transient, rising in 30 s and returning to basal levels within 30 min after a stimulus (6, 15), we measured bacterial killing after 30 min of exposure to monocytes: bactericidal activity was the same in the presence as in the absence of 5-HT or MNNG. Thus, it appears that increasing monocyte cGMP levels is not by itself sufficient to modulate monocyte bactericidal activity.

DISCUSSION

Human monocyte adenylate cyclase is activated by PGE_1 and by cholera toxin. The mechanism by which these agents interact with adenylate cyclase has been studied extensively in other cell types (12, 23). Both agents inhibit the ability of monocytes to kill *S. aureus*. The PGE_1 inhibition could be reversed by inhibiting monocyte adenylate cyclase with RMI 12330A. This agent is not completely specific for adenylate cyclase, having been shown to inhibit two other plasma membrane enzymes (14). However, the fact that RMI 12330A inhibited PGE_1-induced activation of adenylate cyclase and intracellular accumulation of cAMP and also reversed the PGE_1 inhibition of monocyte bactericidal activity supports the hypothesis that cAMP is the mediator of PGE_1 inhibition of monocyte bactericidal activity.

RMI 12330A has been shown to inhibit adenylate cyclase in several other tissues, including rabbit ileum (B. Siegel and N. Wiech, Gastroenterology **70**:79A, 1976), human colon (40), rat liver (13), and spleen, brain, heart, and kidney (14). If RMI 12330A nonspecifically impaired monocyte membrane function, one would expect it to inhibit monocyte bactericidal activity, but by itself RMI 12330A had no effect on bactericidal activity. The mechanism by which cAMP inhibits bactericidal activity is not delineated by this study. cAMP-dependent protein kinase has been demonstrated in macrophage supernatants (36), but the phosphorylated substrate and its function remain unknown. Identification of the substrate for cAMP-dependent protein kinase in monocytes should aid in elucidating the nature of cAMP-mediated inhibition of bactericidal activity. The bactericidal assay measures a multistep process, including phagocytosis and lysosomal enzyme release. Only the latter step appears to be subject to cAMP modulation. Whether the same mechanism is operative in the inhibition of monocyte bactericidal activity and the inhibition of macrophage functions such as tumoricidal activity and plasminogen activator secretion remains to be shown.

Guanylate cyclase activity is also measurable in human monocytes. The inability of 5-HT to activate guanylate cyclase in disrupted cells is not surprising. Several hormones which stimulate cGMP accumulation in intact cells fail to stimulate guanylate cyclase in cell homogenates (24, 27, 44), although a recent report demon-

TABLE 4. *Effect of cGMP agonists on monocyte bactericidal activity*[a]

Agonist	No. of bacteria killed ($\times 10^6$)
Control	7.4 ± 0.5
0.1 mM MNNG	6.4 ± 0.9
0.01 mM MNNG	7.4 ± 1.0
1 μM MNNG	7.5
1 mM 5-HT	7.6 ± 0.7
0.1 mM 5-HT	7.4 ± 0.7
0.01 mM 5-HT	7.3 ± 0.7

[a] A total of 2.5×10^6 monocytes were incubated with 12.5×10^6 *S. aureus* organisms and the indicated agent. At 60 min, samples were withdrawn, cells were lysed, and the numbers of viable bacteria were determined by colony growth. The number of colonies at 60 min was subtracted from the original colony count to determine the percent killing. Values are expressed as the mean ± the standard error of the mean for three experiments. None of the values is statistically different from the control by Student's t test analysis.

strates 5-HT activation of membrane-bound guanylate cyclase in brain tissue (29). NaN_3 and MNNG were able to stimulate intracellular cGMP accumulation and also to activate guanylate cyclase in disrupted cells. According to the yin/yang hypothesis of opposing actions of cAMP and cGMP (50), monocyte bactericidal activity would be potentiated by cGMP. We were not able to demonstrate cGMP-mediated enhancement of monocyte bactericidal activity. In contrast to studies in which 5-HT enhanced monocyte chemotaxis (35), this agent did not enhance monocyte bactericidal activity. MNNG also did not enhance monocyte bactericidal activity, even though it effected a tremendous increase in cGMP levels. One possible explanation for these negative results is that 5-HT and MNNG have other effects which antagonize their effects on cGMP levels (22); however, monocyte viability was not decreased after exposure to either agent. Another argument is that these normal monocytes are already optimally stimulated, whereas cGMP enhancement is seen only in cells defective in bactericidal capacity. A third possibility is that cGMP plays a permissive role in monocyte function, but is not a direct modulator of bactericidal activity.

Similar observations concerning cGMP have been made in lymphocytes (1). In those studies, large increases in cGMP did not effect demonstrable modulation of lymphoblastic transformation. Thus, the precise role of cGMP in immune function remains largely undefined.

A specific inhibitor of guanylate cyclase would be of great value in elucidating whether cGMP plays a role in monocyte function. Although inhibitors have been described (47, 48), their specificity is not yet well defined. Elucidation of the function of cGMP binding proteins, e.g., cGMP-dependent protein kinase, will also aid in defining the role of this nucleotide in monocyte function. The observation that monocyte bactericidal activity can be inhibited by PGE_1 has implication for the modulation of monocyte function by other hormones normally circulating in human peripheral blood or secreted by microorganisms and tumor cells. Investigations of the effects of these agents and of the mechanism by which cAMP modulates bactericidal activity are now in progress.

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