Hemolytic Plaque Formation by Leukocytes in Vitro

CONTROL BY VASOACTIVE HORMONES

KENNETH L. MELMON, HENRY R. BOURNE, YACOB WEINSTEIN, G. M. SHEARER, JERROLD KRAM, and SARA BAUMINGER

From the Division of Clinical Pharmacology, Departments of Medicine and Pharmacology, University of California Medical Center, San Francisco, California 94143, and the Departments of Chemical Immunology and Biodynamics, The Weizmann Institute of Science, Rehovot, Israel

ABSTRACT Histamine, beta-adrenergic amines, and prostaglandins inhibited hemolytic plaque formation by splenic leukocytes from immunized mice. The same agents had previously been shown to prevent both the IgE-mediated release of histamine from human basophils and the immunologically specific cytolytic activity of murine lymphocytes, through stimulation of the production of cyclic AMP in leukocytes. We therefore tested the hypothesis that cyclic AMP might mediate an inhibitory effect of these drugs by comparing the ability of these agents to inhibit plaque formation with their effects on cyclic AMP accumulation in leukocytes. In splenic cells from three mouse strains, the dose-dependent effects of these agents on cyclic AMP correlated with their inhibition of plaque formation. Beta- but not alpha-adrenergic agonists were effective in both systems, and the effects of isoproterenol were inhibited by propranolol. Histamine was approximately equipotent with isoproterenol in both systems. Two prostaglandins (E_1 and E_2) were effective in both systems, but prostaglandin $F_{2\alpha}$ was not. Dibutyryl cyclic AMP, a lipid-soluble analog of the endogenous nucleotide, inhibited plaque formation by cells of all three strains. Theophylline, an inhibitor of cyclic AMP degradation, inhibited plaque formation slightly, but potentiated the effects of histamine, isoproterenol, and the prostaglandins on both cyclic AMP accumulation and plaque formation. Finally, cholera enterotoxin, a potent activator of adenyl cyclase, produced a delayed inhibition of plaque formation and a parallel increase in leukocyte cyclic AMP content; both effects of the toxin were blocked by canine antitoxin. These results suggest that leukocyte cyclic AMP may act as a "second messenger" to suppress plaque formation in vitro. The inhibitory effects of hormones and cyclic AMP on plaque formation are strikingly similar to their effects on in vitro models of immediate and cell-mediated hypersensitivity. The physiologic significance of these findings is not yet known.

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INTRODUCTION

Histamine, catecholamines, and prostaglandins have recently been found capable of modulating the expression of both immediate and cell-mediated hypersensitivity in vitro. These endogenous vasoactive hormones inhibit both the IgE-mediated release of histamine and other mediators of inflammation from human basophils, human and monkey lung, and rat mast cells (1–6) and the immunologically specific cytolytic activity of mouse splenic lymphocytes (6, 7). All three classes of hormones appear to produce their inhibitory effects by stimulating the production of cyclic AMP, which acts in leukocytes and mast cells as an inhibitory "second messenger" (8). The present paper examines the possibility that the same hormones, acting through intracellular cyclic AMP, may modulate a third expression of the immune response: hemolytic plaque formation by splenic leukocytes from immunized mice.

We chose for these experiments an established in vitro model of antibody release, the formation of hemolytic plaques by splenic leukocytes from mice immunized with sheep red cells (SRC).[1] In experiments with three mouse strains, histamine, beta-adrenergic catecholamines, and certain prostaglandins were found to inhibit plaque formation in a dose-dependent fashion that correlated with the ability of the same agents to stimulate accumulation of cyclic AMP in splenic leukocytes. A second messenger

[1] *Abbreviations used in this paper:* MEM, minimal essential medium; PFC, plaque-forming cells; PG, prostaglandin; SRC, sheep red blood cells.

role for cyclic AMP was further suggested by the effects of dibutyryl cyclic AMP, an analog of the endogenous nucleotide, and theophylline, an inhibitor of its enzymatic degradation. Whether the inhibition was due to inhibition of antibody release from cells, intracellular degradation of antibody, or inhibition of antibody synthesis has not been determined.

Cyclic AMP, however, was measured in a heterogenous mixed population of spleen cells, of which only a small percentage (maximum 1%) actually released antibody specific for SRC. The hypothesis that intracellular cyclic AMP inhibited plaque formation was therefore tested with cholera enterotoxin, which produces a delayed rise in cyclic AMP in every mammalian tissue so far tested (9). The toxin has recently been used as a pharmacologic tool to help confirm the role of cyclic AMP in models of immediate and delayed hypersensitivity (6). The present experiments with the toxin strongly support the inhibitory role of leukocyte cyclic AMP on hemolytic plaque formation.

METHODS

Animals and cell preparations. Three strains of mice, C57BL/6, BALB/c, and (BALB/c × C57BL/6)F_1, or BALB/BL, were supplied by the animal breeding house of the Weizmann Institute. The mice were immunized by intraperitoneal injection of 3×10^8 SRC, and spleens were removed on day 6 (for direct plaque or 19S assays) or day 13 (for indirect or 7S plaque assays) after immunization. Rosette assays were done on normal cells or on day 6 or 13 after immunization. The cells were teased from the spleens, strained through stainless steel mesh (200 mesh), and suspended in Eagle's minimal essential medium (MEM) at pH 7.4. Cell counts were done in 2% acetic acid in duplicate with a hemocytometer (variation between duplicates was less than 4%). Incubations of $12–50 \times 10^6$ cells were performed in a final volume of 1 ml MEM with either saline or drugs added to the samples at indicated concentrations. The incubations were carried out in stoppered plastic tubes (Falcon Plastics, Division of B-D Laboratories, Inc., Los Angeles, Calif.) at 37°C for 15 min with constant shaking. The pH of the solutions was checked at the end of the incubations and incubates were discarded if the pH was less than 7.1 or greater than 7.8. The incubates were then appropriately diluted and prepared for either plaque, rosette, or cyclic AMP assays.

Hemolytic plaque assay. Cell plaque assays were performed by modification of the Jerne, Nordin, and Henry agar plate technique (10), in agarose (L'Industrie Biologique Française, S. A. Gennevilliers, France) on 60-mm plastic disposable petri dishes (A/S Nunk, Copenhagen) (11). The plates were incubated at 37°C for 1 h, and then for an additional h in the presence of 1 ml of 1:10 diluted lyophilized guinea pig complement (Grand Island Biological Co., Grand Island, N. Y.). Direct plaque-forming cells (PFC) were counted after the 2-h incubation period. After the PFC had been marked, indirect PFC were developed on the same plate by incubation for 2 additional h in the presence of 0.1 ml of 1:100 diluted rabbit anti-mouse IgG (12).

Drug incubations were always done in duplicate, and triplicate plates were made from each sample, coded, and read separately by at least two investigators. Values are reported as percent inhibition of plaque formation, as compared with controls, which varied from 50 to 150 plaques/plate on different days. If the variation between any duplicates was greater than 9%, the entire experiment was discarded.

In preliminary experiments aliquots of the diluted incubates containing both cells and drugs were added to plain agar plates. When large numbers of samples were read, it was noted that a delay in reading PFC resulted in progressive loss of inhibition by the drugs, particularly when they had been added to the preincubates at low concentrations. Diluting the incubate before plating apparently critically lowered the concentrations of some drugs (e.g., dibutyryl cyclic AMP) so that they were ineffective. Therefore, in all our experiments, drugs were added to both the top and bottom layer of agarose at concentrations precisely matching those in original incubates. Tests of incubates of cells in media containing drugs by the Trypan blue dye method revealed that 85–92% of cells remained viable for the entire period, including the plate-reading time. Viability of control cells was not significantly different from those in experimental tubes.

To determine whether any drugs used in the plaque assay could directly interfere with the function of complement, the following test was performed in quadruplicate, with 1–2 magnitudes higher concentrations of each drug than were used in the actual plaque inhibition experiments. Serial dilutions of goat anti-SRC were made from 1:10 to 1:160. Aliquots of each dilution (50 μl) were spotted on the Petri dishes containing the drug, agar, and all other components used for the assay, except for the spleen cells. After 1 h at 37°C, complement was added and 2 h later lysis of the SRC was checked. None of the drugs influenced the lysis of SRC, even when complement concentrations were 20–40 times less than those used in the actual plaque assays (with spleen cells). Rosette formation assays were performed by the method of Shearer and Cudkowitz (13).

Leukocyte cyclic AMP. Leukocytes in MEM ($12–50 \times 10^6$ cells/ml) were exposed to drugs for 15 min, unless otherwise noted. Incubations were terminated by centrifuging the cells at 800 *g* for 1 min (0°C), after which the supernatant fluid was discarded and the cell button was resuspended in 1.2 ml 5% trichloroacetic acid (TCA). The TCA was extracted with ether and cyclic AMP assayed by the competition-binding assay of Gilman (14), modified as previously described (2). All values reported are the means of duplicate or triplicate drug incubations, differing by not more than 8%. We were not able to measure the cyclic AMP concentrations of the supernates from the incubations. Such studies are now in progress.

Materials. Prostaglandins were obtained from Dr. Peter Ramwell (Alza Corporation, Palo Alto, Calif.) and Dr. John Pike (The Upjohn Co., Kalamazoo, Mich.). Purified cholera enterotoxin, prepared as previously described (15), was obtained from Dr. R. A. Finkelstein (Dallas, Texas). The canine antitoxin was obtained from Dr. John Seale (N. I. H. Standards). All other drugs were obtained commercially (16).

RESULTS

Histamine and beta-adrenergic catecholamines produced a dose-dependent inhibition of plaque formation by splenic leukocytes from BALB/BL mice immunized with

SRC (Fig. 1). In order to test the hypothesis that cyclic AMP was related to that inhibition, we compared the ability of biogenic amines (histamine and adrenergic amines), prostaglandins, and cholera enterotoxin to stimulate accumulation of cyclic AMP and inhibit plaque formation, in parallel experiments with splenic cells from three mouse strains (BALB/BL, BALB/c, and C57BL/6). Each of the agents has been shown to elevate leukocyte cyclic AMP content by stimulation of adenyl cyclase (2, 6–8, 17), the enzyme which converts ATP to cyclic AMP. In other experiments we tested the effects of both theophylline, which inhibits enzymatic degradation of cyclic AMP, and dibutyryl cyclic AMP, which is thought to mimic the effect of the endogenous nucleotide.

Our results pertain to formation of direct (19S) plaques. In preliminary experiments the same drugs appeared to produce inhibition of indirect (7S) plaque formation as well, although the degree of maximal inhibition was less. With the Jerne plaque technique, on any given day with any given pool of spleen cells, the results showed little variation and were reliable. (Parallel experiments showed less than 11% variation and duplicates less than 9% variation.) However, cells from pools made on different days showed the same qualitative effects of drugs but different quantitative changes. For example, propranolol inhibited BALB/BL cells by 7% on one day and 20% on another. Therefore, we have avoided drawing more than qualitative conclusions from any "representative" experiment (e.g., Fig. 1, or columns 1 and 3 of Table I) and have listed such data as separate numbers. Pooling results of experiments from different days revealed that the trends indicated by the representative data were basically consistent. The data have been expressed as percent of change from the control plates. An experiment was discarded if the control plates did not contain at least 50 plaques or if they contained more than 150. By such pooling techniques statistically significant drug-induced alterations could be determined (Table I).

In four experiments none of the drug classes inhibited rosette formation, even when the same concentrations maximally inhibited plaque formation.

Biogenic amines. In all three mouse strains, histamine and the beta-adrenergic catecholamines inhibited plaque formation (Fig. 1, representative data indicating rank order of drug potency, Table II) and stimulated accumulation of cyclic AMP in splenic leukocytes (Table III). Isoproterenol was in general slightly more potent than histamine, both at inhibiting plaque formation and elevating cyclic AMP content of the cells. In one parental strain (BALB/c), higher concentrations of both amines were required to inhibit plaque formation significantly (Table II). The cyclic AMP data (Table III) are not sufficient to document a parallel lack of nucleotide accumulation in BALB/c cells with these two agents.

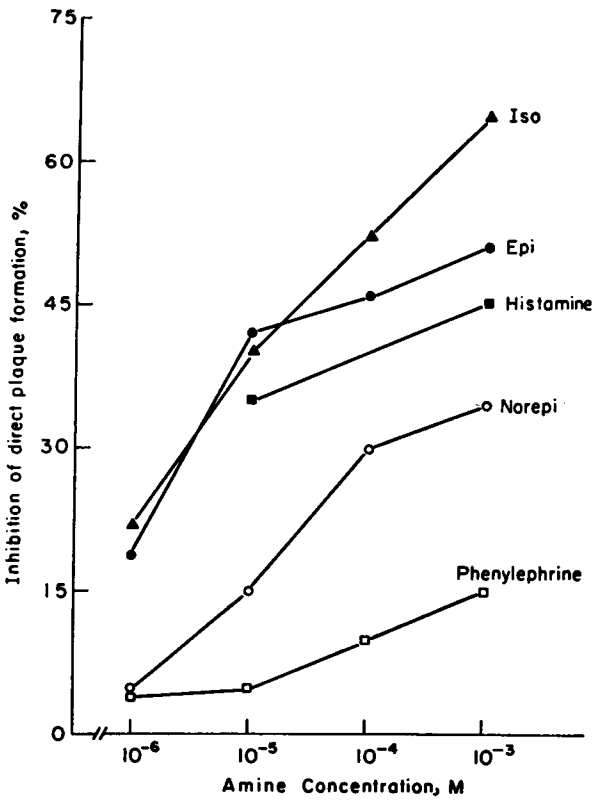

FIGURE 1 Inhibition by amines of 19S (direct) plaque formation of BALB/BL spleen leukocytes. Isoproterenol (▲——▲), epinephrine (●——●), histamine (■——■), norepinephrine (○——○), or phenylephrine (□——□) were added at the indicated concentrations to duplicate leukocyte samples, and plaque formation assessed as described under Methods. Each point represents the average of duplicate drug incubations, differing by not more than 9%.

The relative potencies of the adrenergic agents in all three strains suggested that they produce their effects on cyclic AMP and plaque formation through a beta-adrenergic receptor. The rank order of these amines in inhibiting plaque formation in BALB/BL was exactly parallel to that for inhibition of histamine release from human basophils (2) and for beta-adrenergic effects in other systems (18): Isoproterenol and epinephrine were almost equipotent, norepinephrine was less potent, and the predominantly alpha-adrenergic agent, phenylephrine, was a very weak inhibitor (Fig. 1). In all three mouse strains isoproterenol, a predominantly beta-adrenergic agonist, was considerably more potent than phenylephrine as an inhibitor of plaque formation (Table II) or stimulator of nucleotide accumulation (Table III).

TABLE I

Inhibition of 19S Plaque Formation: Potentiation of Drug Effects by Theophylline

Drug	Concentration	Source of splenic leukocytes				
		C57BL		BALB/c	BALB/BL	
	M					
Theophylline	10^{-2}	4	10	7±17	17	18
Histamine	10^{-5}	28	10	15±6	40	18
+ theophylline	10^{-2}	46	57	40±5	74	46
Isoproterenol	10^{-5}	8	28	21±7	42	36
+ theophylline	10^{-2}	48	57	36±12	69	56
PG E_2	10^{-5}	28	4	30±10	30	8
+ theophylline	10^{-2}	42	44	56±7	64	62

Mean percent decrease in plaque formation. Paired values for C57BL and BALB/BL are the results of experiments on separate days, but all drugs and all three sources of splenic cells were tested on each of the 2 days. Each experimental value represents the mean of duplicate drug incubations, determined from triplicate plates. Values for BALB/c represent the mean percent decrease in plaque formation ±SEM of six experiments. In this strain, the mean values for all three stimulators, in the presence of theophylline, are significantly different from controls and from theophylline alone (P ranges from <0.02 to <0.001 by standard t test).

A beta-adrenergic effect should be blocked by a specific beta-adrenergic antagonist, such as propranolol. In two mouse strains, C57BL and BALB/BL, propranolol completely prevented the effects of isoproterenol (but not histamine) on cyclic AMP accumulation (Table III) and partially blocked isoproterenol's inhibition of plaque formation (Table II). In BALB/c cells, however, propranolol produced significant inhibition of plaque formation when used alone; the inhibition appeared additive to the relatively weak inhibition by isoproterenol. The reason for the different responses of BALB/c spleen cells is not clear. Responsiveness of murine spleen cells to adrenergic agents may be genetically determined, as suggested by species and strain differences documented for other adrenergic responses (19). The propranolol did not significantly change the

TABLE II

Pharmacologic Inhibition of Plaque Formation

Drug	Concentration	Source of spleen cells		
		C57BL	BALB/c	BALB/BL
	M			
Histamine	1×10^{-5}	28±16	0±2	41±16
	1×10^{-4}	46±2	1±7	33±1
	1×10^{-3}	17±7	38±3	36±14
Isoproterenol	1×10^{-6}	28±4	—	18±7
	1×10^{-5}	18±14	3±10	43±7
	1×10^{-4}	54±7	28±2	47±8
Propranolol	1×10^{-5}	6±5	17±6	12±4
Isoproterenol	1×10^{-4}	32±6	19±7	23±8
+ propranolol	1×10^{-5}			
Phenylephrine	1×10^{-6}	4±3	9±7	3±1
	1×10^{-4}	24±5	24±14	16±8
Dibutyryl cyclic AMP	1×10^{-5}	—	46±8	—
	1×10^{-4}	40±8	43±9	50±19

Mean percent inhibition ±SD of five separate experiments. Each experimental value was the mean result of duplicate drug incubations, with plaque counts performed in triplicate.

TABLE III

Effects of Drugs on Cyclic AMP Accumulation in Mouse Splenic Leukocytes

Drug	Concentration	Sources of spleen cells		
		C57BL	BALB/c	BALB/BL
	M			
Control		2.9	<0.5	3.2
Histamine	1×10^{-5}	2.7	<0.5	6.9
	1×10^{-4}	3.9	5.1	6.6
	1×10^{-3}	6.1	9.4	7.8
Isoproterenol	1×10^{-6}	1.2	<0.5	2.7
	1×10^{-4}	11	15	16
Phenylephrine	1×10^{-6}	3.3	<0.5	2.2
	1×10^{-4}	6.0	2.2	6.2
PG E_1	1×10^{-4}	21	15	24
Propranolol	1×10^{-5} M			
+ histamine	1×10^{-3} M	4.3		6.2
+ isoproterenol	1×10^{-4} M	2.0		5.0
+ PG E_1	1×10^{-4} M	26		21

Cells were exposed to drug for 15 min. Each value, expressed as picomoles cyclic AMP per 1×10^7 cells, represents the mean of duplicate determinations, differing by not more than 8%.

cellular cyclic AMP concentrations over control cells in any of the strains tested.

An attempt to block specifically the effect of histamine in BALB/BL cells was unsuccessful, because each of four antihistamines (diphenhydramine, antazolidine, pyribenzamine, and pyrilamine) at $1\text{–}10 \times 10^{-4}$ M significantly inhibited plaque formation when used alone. None of the antihistamines altered the cyclic AMP content of the cells tested.

Prostaglandins. PGE_1 and E_2 were approximately equivalent as stimulators of cyclic AMP accumulation and inhibitors of plaque formation in all three strains (Fig. 2). $PGF_{2\alpha}$ produced no effect on cells from C57BL and BALB/c spleens. A relatively high concentration (1×10^{-4} M) of $PGF_{2\alpha}$ produced a slight rise in cyclic AMP in BALB/BL spleen cells (comparable to the effects of histamine or isoproterenol) and significantly inhibited plaque formation. The pattern of prostaglandin effects on spleen cells from these three mouse strains parallels effects documented in mixed human leukocytes, purified human lymphocytes, human platelets, and other tissues (2, 20, 21): The E prostaglandins are generally potent stimulators of adenyl cyclase, while the F prostaglandins are generally inactive.

Since propranolol failed to block the effect of PGE_1 on cyclic AMP accumulation (Table III), it is likely that the prostaglandins produce their effects through a receptor other than one responsive to beta-adrenergic agents, as has been suggested for human leukocytes (2, 22). For this reason, and because neither the prostaglandins nor the biogenic amines produced complete in-

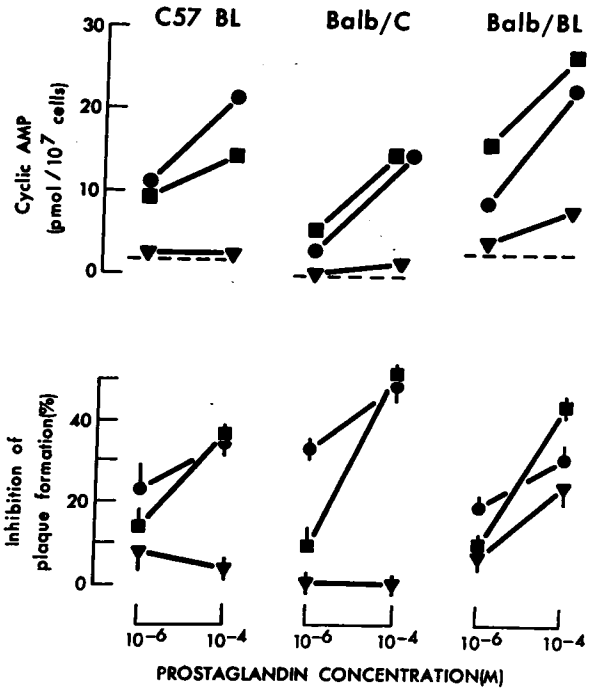

FIGURE 2 Effects of prostaglandins on splenic leukocytes from three mouse strains. Cyclic AMP accumulation (top) and inhibition of 19S (direct) plaque formation (bottom) were measured as described in Methods. The cyclic AMP data represents the mean of duplicate or triplicate determinations, differing by not more than 8%. The plaque inhibition data represents the mean±SD of five experiments, with each drug incubation performed in duplicate. Symbols: PGE_1, ●——●; PGE_2, ■——■; $PGF_{2\alpha}$, ▼——▼.

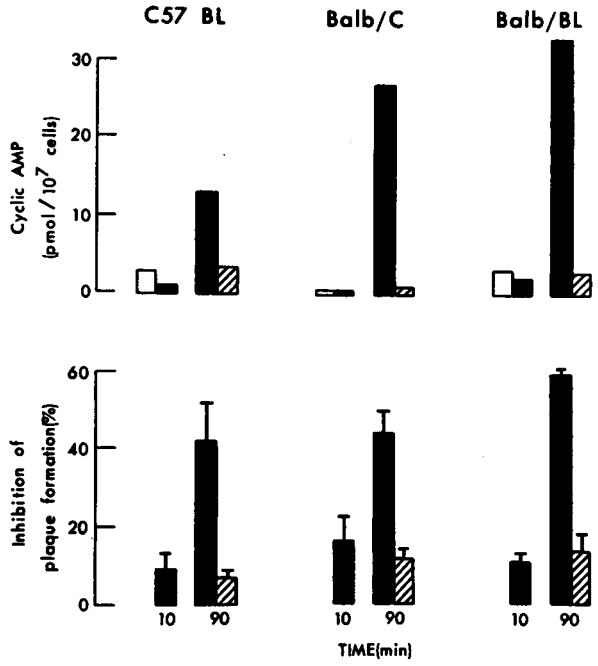

FIGURE 3 Effects of cholera enterotoxin on splenic leukocytes from three mouse strains. Cyclic AMP accumulation (top) and inhibition of 19S (direct) plaque formation (bottom) were measured as described in Methods. Leukocytes were incubated with no drug (open bars), with cholera enterotoxin, 10 ng/ml (black bars), or with cholera enterotoxin, 10 ng/ml, plus antitoxin added simultaneously (1 : 1,000 dilution of canine antiserum, represented by cross-hatched bars). Cyclic AMP data represents the mean of at least duplicate determinations, differing by not more than 8%. Plaque inhibition data represents the mean±SD percent inhibition of five experiments with each drug incubation performed in duplicate. Cholera enterotoxin was added to leukocytes 10 or 90 min before plating, as indicated.

hibition of plaque formation, it seemed possible that antibody-containing cells that contribute to the plaque formation are comprised of subpopulations of cells with different pharmacologic receptors. This would mean that no single class of drug, even at maximally effective concentrations, could completely suppress plaque formation; if this is so, the inhibitory effects of one drug class should be additive to those of another. This proved not to be the case. In BALB/BL spleen cells, no combination of PGE_1, histamine, or isoproterenol produced greater inhibition of plaque formation than the most active drug used alone (results not shown). Consequently, it is likely that the majority of PFCs possess receptors to each of the three drug classes.

Cholera enterotoxin. The choleragenic enterotoxin of *Vibrio cholerae* produces a delayed, dose-dependent activation of adenyl cyclase and accumulation of cyclic AMP in every mammalian tissue so far tested, including human leukocytes and murine splenic lymphocytes (6, 9, 17). The enterotoxin's apparently specific effect on adenyl cyclase, its characteristically delayed effect, and the availability of a specific antitoxin have made it a useful pharmacologic tool for testing hypotheses relating cyclic AMP to function of basophils, neutrophils, and lymphocytes (6, 10). When preincubated for 90 min with spleen cells from C57BL, BALB/c, and BALB/BL mice, cholera enterotoxin (10 ng/ml) inhibited subsequent plaque formation by 42–59% (Fig. 3). By this time, it also produced an increase in cyclic AMP content of splenic leukocytes. A specific canine antiserum (1 : 1,000 dilution) to the toxin prevented both the rise in cyclic AMP and the inhibition of plaque formation at 90 min. A short period (10 min) of preincubation with the toxin failed to affect either cyclic AMP or plaque formation. At these concentrations the toxin did not affect cell viability.

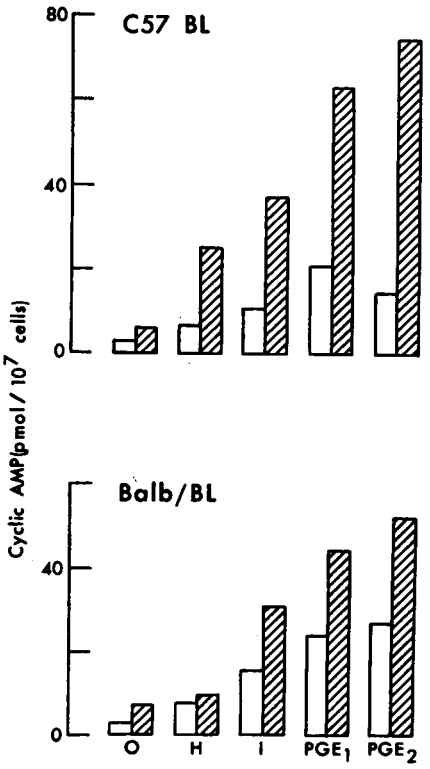

FIGURE 4 Potentiation by theophylline of cyclic AMP accumulation caused by other agents. Cyclic AMP was determined in leukocytes of two mouse strains after 15 min incubation with no drug (O), 1×10^{-4} M histamine, (H), 1×10^{-4} M isoproterenol (I), or PGE_1 and E_2, both at 1×10^{-4} M, and in the presence (cross-hatched bars) or absence (open bars) of theophylline, 1×10^{-3} M. Each bar represents the mean of duplicate determinations, differing by not more than 8%.

Other drugs. In most mammalian systems the hormonal effects mediated by cyclic AMP are reproduced by dibutyryl cyclic AMP. At $1\text{-}10 \times 10^{-5}$ M, dibutyryl cyclic AMP substantially inhibited plaque formation by spleen cells from all three mouse strains (Table II).

The effects of theophylline and other methylxanthines are often adduced as evidence for an effect of cyclic AMP in tissues, since these compounds inhibit the phosphodiesterase that inactivates intracellular cyclic AMP by converting it to 5′-AMP. Theophylline would therefore be expected to potentiate the effects of agents that stimulate adenyl cyclase, in regard to both cyclic AMP content and cell function. Theophylline by itself (1×10^{-3} M) produced little effect on cellular cyclic AMP (Fig. 4) or on plaque formation (Table I) by mouse spleen cells. However, it did potentiate the effects of histamine, isoproterenol, and the prostaglandins on cyclic AMP accumulation (Fig. 4) and plaque formation (Table I).

DISCUSSION

These experiments demonstrate that certain endogenous vasoactive hormones can inhibit plaque formation by leukocytes from three mouse strains. The same hormones in vitro inhibit expression of two other immunologic responses, IgE-mediated immediate hypersensitivity (1–6) and cell-mediated (delayed) hypersensitivity (6, 9), as well as the transformation of human lymphocytes by phytohemagglutinin (20), and the release of lysosomal hydrolases from phagocytic neutrophils (17). Since most of these inhibitory effects appear to be mediated by intracellular cyclic AMP, the experiments reported here were aimed at as critical an evaluation of the role of the nucleotide in inhibiting antibody release as possible. The evidence that cyclic AMP might mediate the hormonal inhibition of antibody in the in vitro system is as follows:

The dose-dependent hormonal stimulation of cyclic AMP accumulation in splenic leukocytes correlated with hormonal inhibition of plaque formation. In each mouse strain the order of potency of both the adrenergic agonists and the prostaglandins was the same for measurement of both cyclic AMP accumulation and inhibition of plaque formation. Propranolol prevented the effect of isoproterenol on cyclic AMP and, in two of the three strains, partially prevented isoproterenol's inhibition of plaque formation. The full interpretation of the effects of propranolol are not possible since the drug itself inhibited plaque formation to some extent, although independently of changes in intracellular cyclic AMP.

A cyclic AMP analog, dibutyryl cyclic AMP, inhibited plaque formation in all three strains.

Theophylline, an inhibitor of enzymatic degradation of cyclic AMP, potentiated the effects of histamine, isoproterenol, and the prostaglandins on both cyclic AMP accumulation and plaque formation.

Cholera enterotoxin, a potent activator of adenyl cyclase in many tissues (9), caused a delayed increase in leukocyte cyclic AMP and a delayed inhibition of plaque formation. Both of these effects were blocked by a specific antitoxin.

The conclusion that cyclic AMP inhibits plaque formation must be qualified by four considerations: first, the formation of a hemolytic plaque is an all-or-none phenomenon, since either enough antibody is available to cause visible hemolysis or no plaque can be recorded. Such an assay system, used to measure what are probably graded inhibitory effects on individual cells, has inherent wide variability, and only the roughest correlations between drug effects and antibody accessibility to erythrocytes can be made. More precise correlations will require sensitive and quantitative assays of the antibody content of cells and supernatant fractions from cell suspensions. Second, the data do not distinguish the mechanisms of inhibition of plaque formation, which could involve inhibition of release or secretion of antibody, or changes in intracellular rates of production or degradation of antibody. Third, our own data indicate that inhibition of plaque formation can occur by more than one mechanism, since antihistamines and propranolol both inhibited plaque formation but failed to alter cellular cyclic AMP content or viability. Fourth, only intracellular cyclic AMP content was measured. Any of the drugs (e.g., antihistamines or propranolol) could have had other effects, including alteration of cell membrane permeability to the cyclic nucleotide. Thus, refinement of these experiments to include measurements of accumulation of cyclic AMP in the extracellular medium might alter their interpretation.

In addition to suggesting a role for cyclic AMP in control of a B-cell–mediated immunologic response, these experiments suggest that antibody-producing cells contain pharmacologic receptors for three classes of vasoactive hormones. In human leukocytes we have shown, using specific pharmacologic antagonists, that the histamine, beta-adrenergic, and prostaglandin receptors are separate, but that at least some of the cells (e.g., basophils) are likely to contain all three receptors (2). The pharmacologic receptors of murine splenic lymphocytes will probably prove to have separate receptors, since propranolol prevented the accumulation of cyclic AMP caused by isoproterenol, but not that caused by histamine or prostaglandins (Table III). The unexplained inhibitory effects of several antihistamines on plaque formation prevented us from examining their effects on cyclic AMP, so that pharmacologic separation of the histamine receptor of these cells from the other receptors remains undefined. Nonetheless, it ap-

pears likely that antibody-producing cells with one receptor contain the other two receptors, since maximally inhibitory effects of the three classes of hormones did not produce additive effects on plaque formation.

We have recently shown that insolubilized histamine and catecholamines attached to Sepharose beads will bind human leukocytes and splenic lymphocytes from the mouse strains used in these experiments (16, 23–25), and that the binding is probably through membrane receptors to the two types of amines. Using the binding phenomenon to separate splenic leukocytes, possibly on the basis of their amine receptors, we have found that the cells that do not bind (presumably those without receptors) also fail to produce plaques on SRC (25). This suggests that the ability to produce specific antibody develops in parallel with the amine receptors, and is consistent with the ability of the free amines to inhibit plaque formation.

The subpopulation of mouse splenic leukocytes with amine receptors is not likely to be confined to cells that produce humoral antibody, but probably includes cells with at least two other immunologic functions: first, splenic cells with "receptors" to histamine, as judged by binding to the insolubilized hormone, have been found to be capable of controlling the humoral antibody response of precursor cells (24). Second, all three classes of hormones used in this study inhibit immunologically specific cytolysis of target cells by murine spleen cells (6, 7) in a model that probably correlates with cell-mediated immunity (delayed hypersensitivity). Two of these three immunologic functions (e.g., cell-mediated immunity and antibody production) are almost surely performed by different cells. Nonetheless, the presence of receptors for amines or prostaglandins or both, acting to regulate the cyclic AMP system, probably is common to cells performing all three functions.

We have not established the details of the biochemical mechanism by which these endogenous hormones and cyclic AMP might regulate immunological function, nor the physiologic significance of these effects in vivo. The almost universally inhibitory effect of cyclic AMP on leukocyte function (8) may indicate that the nucleotide mediates a variety of servomechanisms (negative feedback pathways) that control inflammatory and immune responses in vivo. Present evidence that histamine, catecholamines, and prostaglandins mediate and inhibit inflammatory responses ranges from almost conclusive to fragmentary (26, 27). The impressive interweaving of inflammatory and immune responses in both host defenses and the causation of many diseases suggests, however, that servomechanisms might exist. Both endogenous vasoactive hormones and cyclic AMP may eventually prove to play important roles in controlling or coordinating phenomena as diverse as inflammation, allergy, and humoral and cell-mediated immunity.

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