The Effect of Polystyrene Beads on Cyclic 3′,5′-Adenosine Monophosphate Concentration in Leukocytes

VINCENT MANGANIELLO, WARREN H. EVANS, THOMAS P. STOSSEL, ROBERT J. MASON, and MARTHA VAUGHAN

From the Molecular Disease Branch, National Heart and Lung Institute, and the Laboratory of Biochemistry, National Cancer Institute, Bethesda, Maryland 20014

ABSTRACT After incubation with polystyrene latex beads for 5 min, the cyclic 3′,5′-adenosine monophosphate (cyclic AMP) content of human peripheral blood leukocyte suspensions was increased severalfold. Preparations enriched in mononuclear cells and containing only 0–20% polymorphonuclear leukocytes (PMN) and no visible platelets exhibited a quantitatively similar response. Purified fractions of cells containing 85–90% PMN responded to polystyrene beads with a much smaller increase in cyclic AMP content. Phagocytosis of paraffin oil emulsion in the unfractionated mixed human leukocyte preparation was associated with little or no change in cyclic AMP levels. There was no change in cyclic AMP content of rabbit alveolar macrophages or guinea pig PMN during phagocytosis of polystyrene beads. All of these observations are consistent with the view that particle uptake per se does not increase cyclic AMP levels in phagocytic cells. It seems probable that the increase in cyclic AMP concentration that results when unfractionated human blood leukocytes are incubated with polystyrene beads occurs in cells other than PMN.

INTRODUCTION

Park, Good, Beck, and Davis (1) have recently reported that after incubation with polystyrene latex beads for 5 min, the cyclic 3′,5′-adenosine monophosphate (cyclic AMP)[1] content of human peripheral blood leukocytes is increased severalfold. Since these leukocyte preparations contained a mixture of cell types, and since Stossel, Murad, Mason, and Vaughan (2) had previously found no change in cyclic AMP levels in guinea pig polymorphonuclear leukocytes (PMN) during phagocytosis of polystyrene beads, we investigated the effect of beads on cyclic AMP concentration in PMN purified from human blood. We found that although incubation with beads produced a small increment in cyclic AMP concentration in suspensions of cells consisting of 85–90% PMN, the increase was much less than that observed in similar experiments with unfractionated leukocyte preparations or with fractions of cells containing 0–20% PMN. In addition, phagocytosis of polystyrene beads by rabbit alveolar macrophages was associated with no change in the cyclic AMP content of these cells.

METHODS

Cellular suspensions containing over 90% alveolar macrophages were isolated from normal rabbits and from rabbits which had received an intravenous injection of Freund's adjuvant 21 days before sacrifice (3). Human leukocytes were separated from venous blood obtained in the morning from fasting subjects. For some experiments (designated

Received for publication 14 June 1971 and in revised form 20 September 1971.

[1] *Abbreviations used in this paper:* ACD, acid citrate-dextrose; cyclic AMP, cyclic 3′,5′-adenosine monophosphate; PMN, polymorphonuclear leukocytes; TCA-trichloroacetic acid.

TABLE I

Effect of Phagocytosis on Cyclic AMP Concentration

Exp. No.	Cells source	Cyclic AMP 0 min	Cyclic AMP 5 min	Cyclic AMP 5 min phagocytosis
		pmoles/mg protein		
1	Leukocytes (human)			
	Donor A (heparin)	24.4	30.4	20.9
	B (heparin)	41.3	41.6	72.7
	C (heparin)	27.7	24.2	132
	D (heparin)	11.4	6.7	57.4
2	Leukocytes (human)			
	Donor E (heparin)	23.9	17.4	89.6
	F (heparin)	22.2	13.0	79.2
	E (ACD)	1.8	13.1	161
	F (ACD)	3.7	4.7	219
3	Leukocytes (human)			
	Donor F + G (ACD)	4.3	3.5	105
	F + G (ACD)	4.2	3.2	6.1*
	E (ACD)	4.9	2.1	2.1*
	PMN (guinea pig)‡	2.8	3.8	3.1
4	Alveolar macrophages			
	Normal rabbit	6.7	5.7	5.1
	Normal rabbit	7.1	9.2	8.8
	Treated rabbit	10.1	6.8	7.9
5	Alveolar macrophages			
	Treated rabbit	18.0	14.9	12.6
	Treated rabbit	13.1	14.8	12.2

* These samples of cells were incubated for 5 min with paraffin oil emulsion (4). For all other phagocytosis experiments reported here, cells were incubated with polystyrene beads.

‡ Suspensions containing over 90% PMN were harvested from peritoneal exudates induced in guinea pigs with sodium caseinate (2). The cells were then washed using the same procedure employed with human ACD leukocytes.

heparin in Table I), leukocytes were collected by sedimentation from heparinized blood with dextran, and washed after hypotonic lysis of residual erythrocytes (4). For preparation of leukocytes for gradient separation and in most other experiments, blood was drawn into syringes containing acid citrate-dextrose anticoagulant (ACD) (10 ml NIH formula A/50 ml of blood). Leukocytes were collected and washed as described by Brubaker and Evans (5). A sample of the cells suspended in 0.15 M NaCl was allowed to sediment through a sucrose gradient at unit gravity (5). After removal of samples for total and differential cell counting, cells in each gradient fraction were sedimented by centrifugation at 250 *g* for 5 min and suspended in Hanks' balanced salt solution. Fractions were then pooled, samples taken for differential counts (5), and cells in each pool sedimented again by centrifugation at 100 *g* for 10 min. Cells from a sample of the original cell suspension, not subjected to the gradient separation procedure, were also concentrated by centrifugation before incubation.

Samples of cells in 7.5 ml of Krebs-Ringer phosphate medium were incubated at 37°C for 10 min with agitation in 25-ml siliconized Erlenmeyer flasks. The zero-time sample (2.3 ml) was then taken from each flask and added to 0.5 ml of 30% TCA (trichloroacetic acid) containing 0.25 pmole of cyclic AMP-^{3}H (specific activity 16.3 Ci/mmole), mixed, and placed in a dry ice–ethanol bath. Another sample was transferred to a polyethylene vial containing 0.2 ml of dialyzed polystyrene latex beads (1.1 μ diameter, Dow Chemical Co., Midland, Mich.), and a third to a vial containing 0.2 ml buffer. The final concentration of beads present (approximately 10 mg/ml) provided a bead-cell ratio of at least 100:1 in all experiments. In some experiments 0.2 ml of a paraffin oil–albumin emulsion (4) was used instead of dialyzed beads. After incubation for 5 min, samples were added to tubes containing TCA and were frozen. All samples were then thawed, mixed, and frozen again. After thawing, 0.3 ml 1 N HCl was added, and the mixture was centrifuged. The TCA precipitate was dissolved in 1 N NaOH, and samples were taken for measurement of protein (6).

The supernatant fluid was added to a 0.5 × 6.5 cm column of Dowex-50 (100–200 mesh) previously equilibrated with 0.1 N HCl. After elution with 4.5 ml of 0.1 N HCl and 1.0 ml water, which removed all of the TCA from the column (as evidenced by a spot test sensitive to 0.5 μg TCA [7]), cyclic AMP was eluted with 10 ml water. This fraction was lyophilized, and the residue was suspended in 2.0 ml water and taken to dryness by rotary evaporation under vacuum. Samples were then dissolved in 175 μl water, and portions were assayed at two dilutions for cyclic AMP, according to the method of Gilman (8). With the above elution scheme, depending on the batch of Dowex-50 used, recovery of cyclic AMP-^{3}H varied from 55 to 85% but within any single experiment recoveries varied only 10–20%. In several experiments it was established that incubation of samples with cyclic nucleotide phosphodiesterase (Sigma Chemical Co., St. Louis, Mo.) destroyed 90–95% of the assayable cyclic AMP.

RESULTS

Five of six samples of human peripheral blood leukocytes prepared from heparinized blood (experiments 1 and 2, Table I) exhibited a marked increase in cyclic AMP content after incubation with polystyrene beads for 5 min. In these experiments, the isolation of leukocytes and the incubation conditions, i.e. buffer, temperature, and length of incubation period, were similar to those described by Park et al. (1), and the percentage increase in cyclic AMP content was of the same magnitude as reported by these workers. In all of our experiments, uptake of beads was verified by microscopic examination of washed cells. Phagocytosis of paraffin oil emulsion containing Oil Red O was evaluated in the same way or as previously described (4). A maximal rate of particle uptake was established within the first few minutes of incubation with either substrate for phagocytosis (data not shown).

In experiment 2, Table I, leukocytes from donors E and F were also prepared with ACD. The basal levels of cyclic AMP in these cells were considerably lower, and

TABLE II

Effect of Phagocytosis on Cyclic AMP Concentration in Preparations of Human Leukocytes

Donor	Leukocyte preparation	Cyclic AMP content			Cyclic AMP*	Protein	Differential count‡					Platelets seen
		0 min	5 min	5-min beads			N	M	E	B	L	
		pmoles/mg protein			*pmoles*	*mg*						
E	Unfractionated	7.8	8.8	149	1043	7.0	60	7	3	1	29	+
	PMN enriched	11.6	5.1	27.3	10.9	0.4	92	0	4	0	4	0
	MN enriched§	4.6	4.4	130	208	1.6	20	17	4	0	59	+
F	Unfractionated	7.3	10.2	65.6	558	8.5	64	8	12	1	15	+
	PMN enriched	4.8	3.4	12.5	7.5	0.6	86	4	9	0	1	0
	MN enriched	4.4	3.0	80.6	96.7	1.2	22	23	1	2	52	+
G	Unfractionated	7.4	12.1	93.5	570	6.1	52	10	7	1	30	+
	MN enriched	9.5	4.5	116	128	1.1	0	29	0	1	79	0

* Total amounts of cyclic AMP and protein in the sample of cells incubated with polystyrene beads.
‡ N, neutrophil; M, monocyte; E, eosinophil; B, basophil; L, lymphocyte.
§ MN, mononuclear.

the levels after 5 min with polystyrene beads were higher than those of cells from the same donors prepared without ACD. Phagocytosis of paraffin oil emulsion was associated with little or no change in cyclic AMP content, whereas samples of the same pooled cells from donors F and G responded to polystyrene beads with a 25-fold increase (experiment 3, Table I).

As previously reported (2), phagocytosis of beads by guinea pig PMN was associated with no change in cyclic AMP content (experiment 3, Table I). Alveolar macrophages also, whether from normal rabbits or those injected with Freund's adjuvant, exhibited no alteration in cyclic AMP levels after incubation with polystyrene beads, either for 5 min as shown in experiments 4 and 5, Table I, or for 2–30 min (data not shown).

In the experiments summarized in Table II, peripheral blood leukocytes were separated, and fractions free of platelets consisting of more than 85% PMN were obtained by sedimentation through a sucrose gradient at unit gravity (5). Incubation of the PMN-enriched fraction with polystyrene beads produced a much smaller increment in cyclic AMP content than was observed with the whole unfractionated leukocyte preparation or with the pooled cells, which contained all fractions from the gradient except those included in the PMN-enriched fraction. The pooled fractions, enriched in mononuclear cells, that were incubated with beads contained 2–4 times as much protein as the PMN fraction, but the total cyclic AMP content after 5 min was more than 10 times that in the PMN fractions. In another experiment, cells of donor G recovered from the gradient were pooled to yield a fraction that was enriched in mononuclear cells and contained neither PMN nor platelets. Incubation of this fraction with polystyrene beads produced an increment in cyclic AMP similar to that observed in the total unfractionated leukocyte preparation.

DISCUSSION

We have confirmed the observation of Park et al. (1) that incubation of human peripheral blood leukocytes with polystyrene beads produces a large increment in cyclic AMP content of the cells within 5 min. The increase in cyclic AMP content induced by polystyrene beads in PMN-enriched fractions (no platelets seen) was, however, only a small percentage of that seen with unfractionated leukocyte preparations or with pools of cells from the gradient separation procedure that were enriched in mononuclear cells and contained 0–20% PMN. It will be noted that in one experiment (Table II) with a pool of mononuclear cells from the gradient in which no platelets or PMN were seen, the increment in cyclic AMP concentration was just as great as it was with the unfractionated cells from the same donor. In other experiments (data not shown), incubation of platelet-rich fractions with beads produced a percentage increase in cyclic AMP concentration similar to that seen with the mixed leukocyte preparations. Compared with the latter, however, the total increase in cyclic AMP content of the platelet-rich fractions was small and may well have been due to an effect of the beads on cells other than platelets. There may be some effect of polystyrene beads on the cyclic AMP content of PMN, possibly indirect and dependent on the interaction of beads with another cell type, which would explain the relatively small effect of beads on preparations of purified PMN. It seems probable, however, that incubation of unfractionated leukocytes with poly-

styrene beads produces an increase in the cyclic AMP content of mononuclear cells rather than PMN. Since polystyrene beads do not seem to affect the cyclic AMP content of rabbit mononuclear phagocytes, it is tempting to speculate that the bead-induced increase in cyclic AMP occurs in lymphocytes.

This increase may not be related to phagocytosis of the beads. It has been reported that polystyrene beads stimulate glucose oxidation by Ehrlich ascites tumor cells without being ingested (9). Phagocytosis of paraffin oil emulsion in a suspension of mixed human leukocytes resulted in little or no increase in cyclic AMP content, whereas incubation of the same preparation with polystyrene beads led to a marked increase within 5 min.[3] As reported above and in an earlier publication (2), ingestion of polystyrene beads by guinea pig PMN or rabbit alveolar macrophages was associated with no demonstrable change in cyclic AMP concentration. Although we think it probable that phagocytosis per se does not increase cyclic AMP levels, the nucleotide may well have a role in the control or regulation of this function, as it apparently does in other processes involving microtubule systems. Phagocytosis is inhibited by the addition of dibutyryl cyclic AMP or theophylline to the incubation medium (10, 11). Both of these compounds might be expected to raise the intracellular cyclic AMP concentrations. It would be unwise, however, in the absence of other evidence to conclude that this is the mechanism by which these compounds inhibit phagocytosis. In fact, theophylline interfered with adenosine-stimulated cyclic AMP accumulation in guinea pig cerebral cortex (12), and, depending on the compostion of the incubation medium, exogenous cyclic AMP may either stimulate or inhibit lipolysis in fat cells (13).

[3] Phagocytosis of paraffin oil emulsion by guinea pig PMN produces increases in glucose oxidation, iodination of protein, and incorporation of lysolecithin into lecithin similar to those associated with ingestion of polystyrene beads. Stossel, T., R. Root, R. Mason, P. Elsbach, and M. Vaughan. Unpublished observations.

ACKNOWLEDGMENT

The authors appreciate the excellent technical assistance of Mrs. Betty Hom.

REFERENCES

1. Park, B. H., R. A. Good, N. P. Beck, and R. B. Davis. 1971. Concentration of cyclic adenosine 3',5'-monophosphate in human leukocytes during phagocytosis. *Nature (London)*. **229**: 27.
2. Stossel, T. P., F. Murad, R. J. Mason, and M. Vaughan. 1970. Regulation of glycogen metabolism in polymorphonuclear leukocytes. *J. Biol. Chem.* **245**: 6228.
3. Mason, R. J., G. Huber, and M. Vaughan. 1972. Synthesis of dipalmitoyl lecithin by alveolar macrophages. *J. Clin. Invest.* In press.
4. Stossel, T. P., T. D. Pollard, R. J. Mason, and M. Vaughan. 1971. Isolation and properties of phagocytic vesicles from polymorphonuclear leukocytes. *J. Clin. Invest.* **50**: 1745.
5. Brubaker, L. H., and W. H. Evans. 1969. Separation of granulocytes, monocytes, lymphocytes, erythrocytes and platelets from human blood and relative tagging with diisopropyfluorophosphate. *J. Lab. Clin. Med.* **73**: 1036.
6. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**: 265.
7. Feigl, F. 1956. Spot tests in organic analyses. Elsevier N. V., Amsterdam. 314.
8. Gilman, A. G. 1970. A protein binding assay for adenosine 3',5'-cyclic monophosphate. *Proc. Nat. Acad. Sci. U. S. A.* **67**: 305.
9. Wallach, D. F. H., and M. L. Karnovsky. 1961. Metabolic responses associated with the adsorption of polystyrene spherules by Ehrlich ascites carcinoma cells. Abstracts of the First International Biophysics Congress. Stockholm, Sweden. 43.
10. Stossel, T., R. J. Mason, and M. Vaughan. 1971. A simple sensitive assay for the rate of phagocytosis. *Clin. Res.* **19**: 468.
11. Bourne, H. R., R. I. Lehrer, M. J. Cline, and K. L. Melmon. 1971. Cyclic 3',5'-adenosine monophosphate in the human leukocyte: synthesis, degradation and effects on neutrophil candidacidal activity. *J. Clin. Invest.* **50**: 920.
12. Rall, T. W., and A. Sattin. 1970. Factors influencing the accumulation of cyclic AMP in brain tissue. *Advan. Biochem. Pharmacol.* **3**: 113.
13. Mosinger, B., and M. Vaughan. 1967. The action of cyclic 3',5'-adenosine monophosphate in lipolysis in rat adipose tissue. *Biochim. Biophys. Acta.* **144**: 569.