

Aspirin Effects on Lymphocyte Cyclic AMP Levels in Normal Human Subjects

DIXIE E. SNIDER and CHARLES W. PARKER

From the Department of Medicine, Division of Allergy and Immunology, Washington University School of Medicine, St. Louis, Missouri 63110

ABSTRACT In purified lymphocytes from the peripheral blood of healthy human subjects who had ingested therapeutic doses of aspirin, there was a significant decrease in resting cyclic AMP levels as well as a partial inhibition of the rise in cyclic AMP with isoproterenol or prostaglandin E_1 . These changes were seen as early as 30 min after aspirin ingestion and did not appear to result from aspirin effects on lymphocyte recovery, purity, viability, or relative number of thymus- or bone marrow-derived lymphocytes. In contrast, the direct addition of aspirin to suspensions of purified peripheral lymphocytes did not significantly alter their cyclic AMP levels. However, an effect of aspirin could be obtained in vitro if aspirin was added to unprocessed whole blood during the dextran sedimentation phase of the cell purification. Thus the effect of aspirin on lymphocyte cyclic AMP metabolism may be indirect, through other cells present in the peripheral blood.

INTRODUCTION

During an investigation of peripheral blood lymphocyte cyclic AMP (cAMP) metabolism in normal and asthmatic subjects, cells from a control blood donor were found to have a markedly decreased cAMP concentra-

tion as well as a diminished response to isoproterenol and prostaglandin E_1 (PGE $_1$). After careful questioning, it was determined that she had ingested an ordinary therapeutic dose of aspirin on the same morning the blood was drawn. The present study was undertaken to determine whether aspirin does indeed alter lymphocyte cAMP metabolism and whether the effect can be reproduced by exposing the cells to aspirin in vitro.

METHODS

Chemicals. Acetylsalicylic acid obtained from two commercial sources (Aldrich Chemical Co., Inc., Milwaukee, Wis., and Sigma Chemical Co., St. Louis, Mo.) gave identical results when added to purified lymphocyte suspensions in vitro. In experiments involving the addition of aspirin in vitro to whole blood, the Sigma product was used. Acetylsalicylic acid used for in vivo studies was Bayer brand aspirin tablets (The Bayer Company, Division of Sterling Drug, Inc., New York) (320 mg each) obtained from a local pharmacy. The acetylsalicylsalicylic acid content of the aspirin preparations used in these studies was determined by the method of Bundgaard (1). The Bayer, Sigma, and Aldrich preparations were found to contain 0.007, 0.054, and 0.087% acetylsalicylsalicylic acid, respectively. Isoproterenol, PGE $_1$, sodium salicylate, and acetylsalicylic acid solutions were prepared daily and carefully adjusted to pH 7.4 just before use.

Subjects and lymphocyte purification. 50–500 ml of blood were obtained by venipuncture with heparin¹ at a final concentration of 10 U/ml to prevent clotting. Healthy adult human volunteers who had not taken aspirin or other non-

This work was presented in part at the Annual Meeting, Midwest Section, The American Federation for Clinical Research, 1 November 1973.

Dr. Snider was supported by the U. S. Public Health Service.

Received for publication 8 March 1976 and in revised form 2 June 1976.

¹Liquaemin sodium, Organon Inc., West Orange, N. J., or heparin sodium, Nutritional Biochemicals Corporation, Cleveland, Ohio.

steroidal anti-inflammatory drugs for at least 1 wk before the experiment were used as cell donors. Blood was obtained between 8:00 and 9:30 a.m. and processed as rapidly as possible. Lymphocytes were purified by Ficoll-Hypaque density gradient centrifugation and characterized as previously described (2). Preparations contained 93–98% mononuclear cells, almost all of which appear to be lymphocytes as judged by morphologic and functional criteria. In some experiments, aspirin was added to portions of the blood just after the addition of heparin and remained present during the dextran sedimentation portion of the purification procedure (see below).

Experimental conditions in vitro. Purified lymphocytes were suspended in Gey's solution, pH 7.4, at final concentrations usually ranging from $2\text{--}5 \times 10^6/\text{ml}$. The cells were then incubated for various time periods at 37°C in the presence and absence of stimulatory agents. The incubation conditions were chosen on the basis of previous studies from this laboratory (3). No pH change of greater than 0.2 units was observed during the incubation.

After incubation, the cells were harvested by centrifugation at room temperature for 2 min at 1,500 *g*. Cyclic AMP was determined in the pellets by radioimmunoassay (4). Control experiments excluded possible effects of aspirin or salicylate in the immunoassay.

Exposure to aspirin in vivo. Conditions for exposure to aspirin in vivo in acute and overnight ingestion experiments are as outlined below and in the legend to Table I.

Other studies. Plasma salicylate levels were determined by the method of Trinder (5). Procedures for the enumeration of immunoglobulin-bearing cells with polyvalent rabbit anti-human Ig and fluoresceinated goat anti-rabbit Ig have been described in detail previously (2, 3).

The statistical significance of differences in mean cyclic AMP values was determined by the two-tailed paired *t* test (6).

RESULTS

In our initial experiments, aspirin in final concentrations of 500 and 100 $\mu\text{g}/\text{ml}$ was added in vitro in lymphocyte suspensions preincubated at 37°C for 30 min. The cells were then incubated for an additional 30 min at 37°C . The above aspirin concentrations were chosen because they approximate the upper and lower limits of plasma salicylate concentrations achieved during high- and low-dose aspirin therapy. Under these conditions, there was a very modest and statistically insignificant increase in cAMP in the lymphocytes exposed to aspirin (19 ± 1 , 19 ± 5 , and 14 ± 3 SEM pmol/ 10^7 cells at 500, 100, and 0 μg aspirin/ml, respectively, in six experiments [not shown]). In seven experiments with lymphocytes preincubated with aspirin at 37°C for 60 mins, resting cyclic AMP levels were again unaltered (22 ± 5 , 22 ± 3 , and 17 ± 1 pmol/ 10^7 cells at 500, 100, and 0 μg aspirin/ml, respectively [not shown]). Moreover, when aspirin and isoproterenol or PGE_1 were used in combination, results similar to those with isoproterenol or PGE_1 alone were obtained. Experiments using longer or shorter preincubations, shorter incubations with isoproterenol or PGE_1 (5, 10, or 15 min), or sodium salicylate instead of aspirin also failed to reveal a clearly

TABLE I
Overnight Aspirin Studies

Stimulator (final concentration)	Cyclic AMP levels		<i>P</i> value
	Without aspirin	With aspirin	
	pmol/ 10^7 cells		
None	20 ± 3	8 ± 1	<0.005
Isoproterenol, 10 mM	176 ± 27	98 ± 13	<0.005
PGE_1 , 30 μM	179 ± 34	94 ± 20	<0.02

10 different normal subjects were given 640 mg of aspirin by mouth 12 h and 1 h before venipuncture. Cells were preincubated for 30 min at 37°C and then incubated for an additional 30 min at 37°C in the presence and absence of stimulatory agents. Lymphocyte cyclic AMP levels after aspirin ingestion were compared to control levels in the same subjects (obtained the preceding day in eight subjects and during the preceding week in two subjects). In five subjects the percentage of immunoglobulin-bearing cells was examined before and after aspirin ingestion and found to be unchanged. Results are means \pm SEM.

definable effect on resting or stimulated levels (not shown).

To determine whether the ingestion of aspirin could affect lymphocyte cyclic AMP metabolism, 10 different normal subjects participating in the overnight aspirin study were given two aspirin tablets (640 mg) 12 h and 1 h before venipuncture (a total of 1,280 mg). In three subjects this dose of aspirin produced plasma salicylate levels of 4.5–6.2 mg/100 ml at the time of venipuncture. As shown in Table I, mean resting and stimulated cyclic AMP levels after aspirin ingestion were approximately 50% of the control levels, although the percent increases in response to isoproterenol and PGE_1 after aspirin ingestion were similar to that of the controls.

Although the foregoing studies had demonstrated that aspirin ingestion could affect lymphocyte cyclic AMP levels, it was not clear how rapidly this effect occurred. Therefore, 13 different normal fasting subjects participating in the acute aspirin study were given three aspirin tablets (960 mg) by mouth with water. Salicylate levels were measured in three of these subjects and were found to range from 0.2 to 5.1 mg/100 ml at 30 min and from 1.5 to 5.1 mg/100 ml at 60 min. (All salicylate levels were zero at zero time).

In the zero-time cells, the mean cyclic AMP levels after incubation with buffer, PGE_1 , or isoproterenol under the conditions in Table I were consistently higher than in similarly treated cells obtained 30 min after ingestion of aspirin (*P* values of <0.02 for unstimulated 1 mM isoproterenol, and 30 and 3 μM PGE_1 ; <0.05 for 10 mM isoproterenol, not shown). By 60 min the responses to both concentrations of isoproterenol were

no longer significantly decreased, but the PGE₁ responses remained depressed. The possibility that the decreases were in some way related to repeated venipuncture is quite unlikely, since we failed to observe these changes in six similarly studied subjects who had not ingested aspirin. Similar control studies also produced negative results in the overnight experiments. In addition, no evidence was obtained that exposure to aspirin in vivo affected lymphocyte recovery or purity or B cell-T cell ratios.

Because the in vivo effect of aspirin on lymphocyte cyclic AMP metabolism could not be reproduced by adding aspirin, sodium salicylate, or both (two experiments, not shown) directly to suspensions of purified human peripheral lymphocytes, additional in vitro experiments were performed in which aspirin was added to whole blood in quantities calculated to give final concentrations of 100 µg/ml or 500 µg/ml during the dextran sedimentation step (Table II). In cells exposed to the higher (500 µg/ml) concentration of aspirin during dextran sedimentation, significant decreases in cAMP under all three experimental conditions (buffer, PGE₁ isoproterenol) were obtained. At the lower (100 µg/ml) aspirin concentration, cyclic AMP levels were not statistically decreased, although some diminution in the mean response was obtained with isoproterenol and PGE₁.

DISCUSSION

The results presented in this paper provide evidence that exposure of human beings to ordinary therapeutic doses of aspirin results in alterations in peripheral lymphocyte cAMP levels that persist even after the cells have been extensively purified. The absolute response to stimulation with isoproterenol and PGE₁ was also depressed after aspirin ingestion, although the relative (percent) increase above resting levels was similar to that of cells not exposed to aspirin. Interestingly, this aspirin effect was not seen with direct additions of aspirin to purified lymphocytes in vitro. However, when aspirin was added to whole blood in vitro, a suppressive effect was demonstrated, although only at the higher aspirin concentration. It is therefore tempting to speculate that aspirin may be stimulating the release of a soluble mediator from nonlymphocytic cells, which in turn alters cAMP levels in lymphocytes, probably by altering their adenylate cyclase or phosphodiesterase activity.

The ability of aspirin to alter lymphocyte cAMP levels raises the interesting issue of whether similar effects of aspirin are occurring in other tissues. In particular, it is worth considering the possible role of analogous alterations in cAMP metabolism in the lung in the pathogenesis of aspirin-sensitive asthma. There is at least one report of aspirin-induced alterations in adenylate cyclase activity in the rat gastric mucosa (7), but by

TABLE II
Effects of Having Aspirin Present during the Dextran Sedimentation Phase of Cell Purification on Subsequent Lymphocyte Cyclic AMP Levels

Stimulator (final concentration)	Cyclic AMP level		
	Without aspirin	With aspirin	
		500 µg/ml	100 µg/ml
		pmol/10 ⁷ cells	
None	14 ± 2	8 ± 4 (<0.05)*	15 ± 5 (>0.7)
Isoproterenol, 10 mM	101 ± 21	64 ± 17 (<0.05)	88 ± 29 (>0.05)
PGE ₁ , 10 µg/ml	144 ± 21	95 ± 13 (<0.05)	119 ± 28 (>0.3)

Heparinized blood (200–500 ml) was obtained from single donors and divided into four or six equal parts. Aspirin was dissolved in PBS, pH 7.4, at a concentration of 2 mg/ml. Volumes of this solution calculated to give final aspirin concentrations of 500 or 100 µg/ml were added to duplicate batches of cells. Control cells were treated in duplicate with equivalent volumes of phosphate-buffered saline. The aspirin and phosphate-buffered saline remained present during the entire dextran sedimentation step. Salicylate levels of 45–98 µg/ml and 240–270 µg/ml, respectively, were obtained at the termination of the sedimentation step, indicating that at least one half of the added aspirin had been hydrolyzed during sedimentation. Subsequently cells were purified in the usual manner with aspirin-free solutions. Incubation conditions are given in the legend to Table I.

* P value derived from two-tailed paired *t* test.

and large this subject has received very little attention. As far as lymphocytes themselves are concerned, a number of investigators have reported that exposing these cells to aspirin either in vitro or in vivo can inhibit antibody synthesis or lymphocyte transformation responses to lectin (for example, refs. 8–10). Whether or not cAMP is involved in these effects is a subject for further investigation.

ACKNOWLEDGMENTS

This investigation was supported by Allergy Clinical Center Grant 5P15 AI10405 and by National Institutes of Health Training Grant AI00219.

REFERENCES

1. Bundgaard, H. 1976. Colorimetric analysis of immunogenic impurities in acetylsalicylic acid. *J. Pharm. Pharmacol.* In press.
2. Eisen, S. A., H. J. Wedner, and C. W. Parker. 1972. Isolation of pure human peripheral blood T lymphocytes using nylon wool columns. *Immunological Communications*. 1: 571–577.
3. Parker, C. W., and J. W. Smith. 1973. Alterations in cyclic adenosine monophosphate metabolism in human bronchial asthma. I. Leukocyte responsiveness to β -adrenergic agents. *J. Clin. Invest.* 52: 48–59.
4. Steiner, A. L., D. M. Kipnis, R. Utiger, and C. W. Parker. 1969. Radioimmunoassay for the measurement of adenosine 3':5'-cyclic phosphate. *Proc. Natl. Acad. Sci. U. S. A.* 64: 367–373.
5. Trinder, P. 1954. Rapid determination of salicylate in biological fluids. *Biochem. J.* 57: 301–303.
6. Hill, A. B. 1961. Principles of Medical Statistics. Ox-

- ford University Press, Inc., New York. Seventh edition. 149-151.
7. Mangla, J. C., Y. M. Kim, and A. A. Rubulis. 1974. Adenyl cyclase stimulation by aspirin in rat gastric mucosa. *Nature (Lond.)*. **250**: 61-62.
 8. Panush, R. S., and C. R. Anthony. 1976. Effects of acetylsalicylic acid on normal human peripheral blood lymphocytes. Inhibition of mitogen- and antigen-stimulated incorporation of tritiated thymidine. *Clin. Exp. Immunol.* **23**: 114-125.
 9. Crout, J. E., B. Hepburn, and R. E. Ritts. 1975. Suppression of lymphocyte transformation after aspirin ingestion. *N. Engl. J. Med.* **292**: 221-223.
 10. Ambrose, C. T. 1966. Inhibition of the secondary antibody response *in vitro* by salicylate and gentisate. *J. Exp. Med.* **124**: 461-482.