The Effect of Salicylate on the Metabolism of Normal and Stimulated Human Lymphocytes In Vitro

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ABSTRACT The effect of salicylate on the metabolism of peripheral blood lymphocytes in tissue culture was investigated. Lymphocytes incubated with sodium salicylate at a concentration of 30 mg/100 ml showed increased glucose consumption, lactic acid production, and oxygen consumption, evidence for uncoupling of oxidative phosphorylation. No decrease in cell number or viability (trypan blue dye exclusion) was noted in salicylate-treated cultures. Normal DNA, RNA, and total protein synthesis measured by radioisotope incorporation was depressed in the salicylate-treated cultures. Increased DNA synthesis after the addition of a mitogen (PHA) or antigen (PPD) to the culture was strikingly suppressed by salicylate. The degree of suppression was proportional to the concentration of salicylate used. The effect on RNA and protein synthesis in stimulated lymphocytes was much less pronounced. Acetylsalicylic acid was found to be as active as sodium salicylate in suppressing DNA synthesis, but the *p*-OH congener (*p*-OH benzoic acid) did not alter cell respiration, glycolysis, viability, or DNA synthesis. The salicylate effect was reversible as evidenced by return of cellular reactivity upon removal of the drug from the media.

INTRODUCTION

Salicylates are the treatment of choice for rheumatoid arthritis and rheumatic fever without carditis (1, 2), although their mechanism of action remains unknown. After ingestion, aspirin is rapidly hydrolyzed to the free acetyl radical and salicylate (3-6). Blood levels of

salicylate are monitored and considered therapeutic at concentrations of 20-30 mg/100 ml, and symptomatic relief is usually obtained.

Pharmacological studies of the effect of salicylate on various tissues and mitochondria indicate that it uncouples oxidative phosphorylation (7-11), affecting primarily aerobic phosphorylation (12). Previous investigation of the carbohydrate metabolism of lymphocytes, one of the components of the inflammatory cycle, has shown that their oxidative phosphorylation could be uncoupled by 2,4-dinitrophenol (13). The present study was, therefore, initiated to determine some of the effects of salicylate on the metabolism of normal and stimulated human lymphocytes in vitro.

METHODS

Preparation of lymphocytes

Heparinized (10 U of Liquaemin sodium [Organon Inc., West Orange, N. J.] per ml) peripheral blood was obtained from healthy adult human donors as previously outlined (13). Gentran 6% w/v in 0.9% sodium chloride (Dextran 70, Travenol Laboratories, Inc., Morton Grove, Ill.) was added to a final concentration of 0.6% and the blood-dextran mixture was sedimented in an inverted syringe at 37°C. The leukocyte-rich plasma layer was removed and applied to a column consisting of an equal volume of absorbent cotton (Red Cross, Johnson & Johnson, New Brunswick, N. J.). After elution with Hank's solution, the effluent, containing mononucleated cells, red cells (not more than a 3:1 ratio), and no more than 1% polymorphonuclear leukocytes, was concentrated and suspended in media.

Conditions of culture

In short-term experiments measuring respiration, the cells were suspended in phosphate buffer containing 1000 µg of glucose per ml at a concentration of 2.5×10^7 /ml, and maintained at 37°C.

In long-term experiments, lasting 3 or 5 days, the lymphocytes were suspended in complete Eagle's Minimal Essential Medium spinner modification (CMEM, all components from Gibco, Grand Island, N. Y.) containing 20% heat-inactivated

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TABLE I
In Vitro Effects of Sodium Salicylate and p-OH Benzoic Acid on Lymphocyte Respiration, Glycolysis, and Viability

Freshly isolated cells			After 3 days in vitro					Per cent Trypan blue negative
Addition	Respiration*	P	Glucose‡	P	Lactic acid‡	P	Cell count§	
Saline	3.05 ±0.15 (7)¶		1.56 ±0.31 (8)		2.49 ±0.16 (8)		1.99 ±0.085 (10)	83.1 ±1.50 (10)
p-OH Benzoic Acid, 30 mg/100 ml	3.02 ±0.12 (3)	>0.50	1.90 ±0.56 (8)	>0.50	2.76 ±0.34 (8)	>0.40	—	—
Sodium salicylate, 30 mg/100 ml	4.13 ±0.12 (7)	<0.001	4.08 ±0.54 (8)	<0.005	3.50 ±0.47 (8)	<0.05	1.81 ±0.57 (10)	80.3 ±1.30 (10)
30 mg/100 ml of sodium salicylate followed by 2 × 10 ⁻⁴ M KCN	0.46 ±0.06 (3)		—		—		—	—

The effect of sodium salicylate and p-OH benzoic acid on the oxygen consumption of freshly isolated lymphocytes and on cellular glycolysis and viability after 3 days in vitro.

* μ moles of O₂ per 10⁷ per min; RBC present in not more than 1:1 ratio.

‡ Change in concentration in mmoles/10⁷ cells.

§ Times 10⁴ cells per culture.

|| Mean ±SE.

¶ Number of experiments, triplicate observations in each.

fetal calf serum (56°C for 30 min), 2 mmoles/ml of L-glutamine, 50 U/ml of penicillin, and 50 μ g/ml of streptomycin. The cell suspension was adjusted to a final concentration of 2.5–3.0 × 10⁶/ml and dispensed into 16 × 100 mm disposable flint glass tissue culture tubes (Bellco Glass, Inc., Vineland, N. J.). The cultures were maintained with loosely fitted caps (Kaput, Bellco) in a 5% CO₂-95% air incubator at 37°C for the appropriate time periods. Phenol red-free media was used in experiments in which glucose, lactic acid, or salicylate determinations were performed.

Experimental design

Freshly isolated lymphocytes in high concentrations were used to evaluate the immediate effect of salicylate and p-OH benzoic acid on respiration. In longer-term studies, lower cell concentrations were prepared in triplicate for each additive. After three days in vitro, total cell number and viability was determined, and the supernatant media obtained for glucose, lactic acid, and salicylate content.

The effect of salicylate on lymphocyte nucleic acid and protein synthesis in vitro was measured in duplicate cultures with and without the simultaneous addition of mitogen (PHA or PPD). In experiments using PHA, the appropriate tritiated precursor was added to the cultures on day 1, 2, and 3; in those using PPD, the label was added on day 1, 3, and 5.

Determination of cell viability

Cell viability was assessed by exclusion of 1% trypan blue dye (13). 50 cells were counted from each of quadruplicate tubes containing either salicylate-treated or control cells. The slides were coded during preparation and evaluated independently by two observers. Total white blood cell counts were performed using Hayem's solution and a standardized Neubauer hemocytometer.

Additions to cultures

Salicylates. Sodium salicylate (Matheson, Coleman & Bell, Norwood, Ohio) was dissolved in phosphate-buffered saline (PBS) and added to the cell suspensions in the stated concentrations. Acetylsalicylic acid (Aspirin, Matheson, Coleman & Bell) 30 mg/100 ml in PBS or parahydroxide benzoic acid (p-OHBA, Aldrich Chemical Co., Inc., Milwaukee, Wis.) 30 mg/100 ml in PBS were used in the experiments as indicated. The pH of the starting media was unaltered by the concentrations of sodium salicylate used; after addition of p-OH benzoic acid or aspirin, the pH was adjusted to 7.4. The concentration of salicylate in the media was determined by the method of Trinder (14).

Mitogenic agents. Phytohemagglutinin (PHA, Burroughs Wellcome & Co., London, England, lot No. 9312) was used as a nonspecific mitogen in a final concentration of 20 μ l/ml of tissue culture media. Purified tuberculin protein (PPD, supplied by Dr. Fisher of Parke, Davis & Co., Detroit, Mich.) was used as an antigenic stimulus in a final concentration of 10 μ g/ml to achieve maximal stimulation of lymphocytes from previously assayed skin test positive donors.

Chemical determinations

Oxygen consumption. Oxygen consumption was determined in a microcuvette with a Clarke Oxygen Electrode (Gilson Medical Electronics Inc., Middleton, Wis.) and expressed as μ moles/10⁷ cells per min (13). The drugs were added to the microcuvette and the effect on cell respiration was recorded over a 10 min period.

Glucose consumption and lactic acid production. Glucose levels were determined (Glucostat, Worthington Biochemical Corp., Freehold, N. J.) and the lactic acid content of the media was measured (Stat pack, Calbiochem, Los Angeles, Calif.) at the beginning and the end of the culture period,

and the change in each was expressed as mmoles/10⁷ cells (13).

DNA synthesis. 1 μ Ci of methylthymidine-³H, specific activity 1.9 Ci/mmmole (Schwarz Bio Research Inc., Orangeburg, N. Y.), was added to each culture on the designated day. After 4 hr, the culture tubes were centrifuged at 150 g for 10 min at 4°C and the supernatant was removed. The cells were frozen and thawed once and extracted for DNA twice with cold 5% trichloroacetic acid (TCA) and twice with cold absolute methanol. Each precipitate was dissolved in 0.3 ml NCS solubilizer (Nuclear-Chicago, Des Plaines, Ill.) and 10 ml of scintillator, Liquifluor (New England Nuclear Corp., Boston, Mass.), containing 6 g of 2,5-diphenyloxazole and 0.075 g of *p*-bis 2-(5-phenyloxazoly) benzene per liter of toluene. The radioactivity of the samples was measured in a Packard Tricarb model No. 3320 (Packard Instrument Co., Inc., Downers Grove, Ill.).

RNA synthesis. 5 μ Ci of 5-uridine-³H, specific activity 4 Ci/mmmole (Schwarz Bio Research), were added to each culture for a 4 hr period as indicated. The cells were recovered, frozen, and thawed, and bovine serum albumin (BSA, Armour Pharmaceutical Co., Chicago, Ill.) 500 μ g/tube, was added as carrier. RNA was extracted according to the method of Kay and Korner (15); the final precipitate was dissolved in NCS and the radioactivity measured as above.

Total protein synthesis. Lymphocytes were cultured in leucine-free CMEM (Gibco) and each tube was pulsed with 5 μ Ci of 4,5-leucine-³H, specific activity 6 Ci/mmmole (Schwarz Bio Research). After 4 hr, the cells were recovered, BSA 500 μ g/tube was added as carrier, and the protein extracted, according to the method of Schneider (16), solubilized with NCS, and the radioactivity determined.

RESULTS

Respiration, glycolysis, and viability. Salicylate increased the respiration of freshly isolated lymphocytes by 31%; this stimulation was inhibited by cyanide, leaving a cyanide-resistant respiration of 15% (Table I). No change in lymphocyte oxygen consumption was observed with addition of comparable concentrations of *p*-OH benzoic acid. Salicylate, but not *p*-benzoic acid,

increased cellular glucose consumption by 161%, and lactic acid production by 40% after 3 days in vitro without altering the viability or total number of mononuclear cells.

Nucleic acid and protein synthesis. The suppressive effect of salicylate on DNA synthesis in control cultures, and those stimulated with PHA or PPD was dose related (Table II). The levels of salicylate in the test media remained constant throughout the culture period. Individuals varied in their degree of sensitivity to salicylate, but multiple determinations on consecutive cell samples from a given donor were consistent. Aspirin in a concentration of 30 mg/100 ml suppressed DNA synthesis. A similar concentration of *p*-OH benzoic acid was without effect.

RNA and total protein synthesis in control cells, and those stimulated by PHA or PPD were measured daily (Table III). DNA synthesis was determined on day 3 from PHA-treated cells and on day 5 for antigen-sensitive cells exposed to PPD. In vitro concentrations of 30 mg/100 ml of salicylate inhibited DNA synthesis by 94% in both PPD- and PHA-stimulated cells. RNA and total protein synthesis were both suppressed to a lesser extent in cells responding to either PHA or PPD.

The effect of salicylate on RNA synthesis was reversible, as seen in Table IV, which shows data from one of four experiments. The initial culture media containing PHA, salicylate, PHA and salicylate, or saline alone was removed after 24 hr, and replaced with fresh media containing either 30 mg/100 ml of sodium salicylate or saline. Decrease in incorporation of tritiated uridine 48 hr later (after a total of 3 days in vitro) was dependent on the continued presence of salicylate in the media. Removal of salicylate from the media resulted in RNA synthesis equal to that found in cultures treated with PHA alone.

TABLE II
Suppression of Stimulated Lymphocyte DNA Synthesis In Vitro by Salicylate

Additions to culture	Sodium salicylate				Aspirin	<i>p</i> -OH benzoic acid
In vitro concentration mg/100 ml	5	10	20	30	30	30
Mean per cent suppression of PPD stimulated cells	45.1 $\pm$ 7.1* (3)†	69.4 $\pm$ 4.6 (3)	81.5 $\pm$ 3.7 (4)	94.3 $\pm$ 1.8 (5)	89.7 $\pm$ 3 (3)	12.9 $\pm$ 9.5 (3)
Mean per cent suppression of PHA stimulated cells	—	56.7 $\pm$ 0.3 (3)	74.8 $\pm$ 4.3 (4)	94.4 $\pm$ 1.6 (8)	83.7 $\pm$ 4.6 (6)	7.0 $\pm$ 4.8 (3)

The effect of various doses of salicylate on in vitro DNA synthesis assayed by uptake of tritiated thymidine in cells stimulated by PHA (measured after 3 days of culture) or PPD (measured after 5 days in vitro).

* Mean $\pm$ SE.

† Number of experiments, triplicate observations in each.

TABLE III
Salicylate Suppression of Lymphocyte Nucleic Acid and Protein Synthesis In Vitro

Substance added	Mean per cent suppression by salicylate of:	Days in culture			
		1	2	3	5
PHA	Total protein	49.50 $\pm$ 9.4* (4)†	38.40 $\pm$ 3.9 (5)	62.50 $\pm$ 1.2 (4)	—
"	RNA	36.70 $\pm$ 10.5 (4)	54.14 $\pm$ 6.2 (7)	65.20 $\pm$ 5.0 (8)	—
"	DNA	—	—	94.55 $\pm$ 1.6 (8)	—
PPD	Total protein	20.80 $\pm$ 2.4	—	51.12 $\pm$ 6.6	79.54 $\pm$ 3.5
"	RNA	26.34 $\pm$ 7.3 (3)	—	57.13 $\pm$ 7.2 (4)	80.82 $\pm$ 3.4 (4)
"	DNA	—	—	—	94.28 $\pm$ 1.8 (5)
Saline	Total protein	22.75 $\pm$ 3.8 (8)	24.25 $\pm$ 7.6 (4)	31.53 $\pm$ 4.2 (8)	70.24 $\pm$ 4.2 (4)
"	RNA	12.94 $\pm$ 5.9 (8)	7.14 $\pm$ 12.8 (7)	29.00 $\pm$ 7.8 (8)	57.5 $\pm$ 3.4 (4)
"	DNA	—	—	79.96 $\pm$ 2.5 (8)	78.15 $\pm$ 3.9 (4)

The suppression by in vitro concentrations of 30 mg/100 ml of sodium salicylate of DNA synthesis (measured by uptake of tritiated thymidine), RNA synthesis (measured by uptake of tritiated uridine), and total protein synthesis (evaluated by uptake of tritiated leucine) in cells treated with PHA (assayed on day 1, 2, and 3) or PPD (assayed on day 1, 3, and 5) and their controls.

* Mean $\pm$ SE.

† Number of experiments, duplicate observations in each.

DISCUSSION

Salicylate affects aerobic oxidative phosphorylation in differing ways, depending on the source of the tissue or the mitochondrial preparation (9–12). In the present study, the increased respiratory rate of lymphocytes after the addition of salicylate was diminished to endogenous levels by cyanide, suggesting that salicylate stimulated aerobic-dependent activity. No change in respiration or glycolysis followed the use of the para-substituted congener; ortho-substitution may be a concomitant of chemical activity (17).

Salicylate did not diminish the viability of the number of cells remaining after 3 days in vitro, a finding consistent with shorter term in vitro studies (18, 19), as well as clinical observations.

The tissue culture media in this study contained fetal calf sera, and spontaneous synthesis of cellular nucleic acid and protein was called "normal." Salicylate diminished DNA synthesis in both "normal" and stimulated cells to a greater extent than RNA synthesis. This difference in suppression may reflect a greater sensitivity of DNA versus RNA polymerase to salicylate (20).

Salicylate reduced the incorporation of leucine into protein of human lymphocyte, as well as diaphragm and cell-free liver preparations from the rat (21).

Salicylate altered lymphocytes response to stimulation in a reversible manner; removal of the drug from the media allowed cells treated with PHA to reach the

TABLE IV
Recovery of Lymphocyte Response to PHA as Measured by RNA Synthesis

	Additions to media	
	0–24 hr	24–72 hr
Saline		cpm/culture*
	Saline	9149 $\pm$ 1487
Salicylate†	Saline	8842 $\pm$ 751
	Saline	10,208 $\pm$ 1640
PHA	Saline	8544 $\pm$ 1638
	Saline	111,811 $\pm$ 15,570
PHA	Saline	67,637 $\pm$ 3694
	Saline	149,371 $\pm$ 17,855
Salicylate	Salicylate	68,405 $\pm$ 7012

The recovery of lymphocyte response as measured by RNA synthesis. The media containing PHA, alone or with salicylate, salicylate, or saline alone was removed after 24 hr, and fresh media containing salicylate or saline was added (see text). The RNA synthesis of the treated cells was measured by uptake of tritiated uridine on the 3rd day of culture.

* Mean $\pm$ SE.

† Sodium salicylate 30 mg/100 ml final concentration.

same level of RNA synthesis as found in those cultures to which PHA alone had been added. Reversible competition with NADP or respiratory dehydrogenases have been postulated as possible mechanisms of action of salicylate (22).

Addition of salicylate to leukocyte cultures decreased the number of mitosis (23) without inducing chromosomal aberrations (24). Conflicting data is available concerning the in vitro responsiveness of lymphocytes obtained from patients on therapeutic doses of salicylate (25).¹ This investigation provides evidence that salicylate accelerates respiration and glycolysis of peripheral blood lymphocytes and reduces nucleic acid and protein synthesis in vitro without affecting cell viability. The effect of salicylate on other cellular components of the reticuloendothelial system is currently under investigation (26).

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