Brief Definitive Report

EFFECT OF SODIUM BUTYRATE ON LYMPHOCYTE ACTIVATION*

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Butyrate, in relatively low concentrations, has been shown to induce synthesis of enzymes, cause changes in cell morphology, and inhibit growth of a variety of mammalian cells in tissue culture (reviewed in [1]).

In this communication, we report our observations on the effect of butyrate on lymphocyte activation. Butyrate completely and reversibly inhibits mitogen-induced blast formation. We present evidence that it does not interfere with the binding of mitogens, that it does not inhibit a number of the "early" reactions involved in activation, and that it does not affect ongoing DNA synthesis for an extended period of time. However, butyrate rapidly inhibits any increase in the rate of DNA synthesis.

Materials and Methods

Mouse spleen lymphocytes were isolated from 18–25 g (6–9-wk-old) male or female Swiss Webster mice, by the method of Bøyum (2). Freshly isolated lymphocytes, from the spleen of a single mouse, were incubated either in RPMI-1640 or minimum essential medium. All media were supplemented with 10% heat-inactivated fetal calf serum and 10^{-5} M 2-mercaptoethanol. Two separate duplicate cultures were prepared for each experimental point. Each culture consisted of $1.0–1.5 \times 10^6$ cells in 1 ml medium in a 12×75-mm plastic tissue culture tube maintained for the indicated time at 37°C, with an atmosphere of 5% CO_2. Butyric acid (neutralized with NaOH) was used in 2 mM final concentration or as indicated in Table I.

To measure the rate of DNA synthesis, cells were labeled with 1 or 2.5 μCi/ml [^{3}H]thymidine (sp act: 60 Ci/mmol) for 2 h, filtered onto Millipore membrane filters (pore size: 0.45 μm), washed with 2×1 ml Dulbecco's phosphate-buffered saline minus Ca^{++} and Mg^{++} (PBS), and with 3×5 ml 5% TCA. Acid insoluble radioactivity on dried filters was determined in a liquid scintillation counter. All experiments were repeated a minimum of five times. The data presented are from typical experiments.

Materials. Phytohemagglutinin HA16 (PHA), Burroughs Wellcome & Co., Greenville, N. C.; lipopolysaccharide (LPS), Escherichia coli, 0111:B4, Difco Laboratories, Detroit, Mich.; fetal calf serum (FCS), Microbiological Associates, Bethesda, Md.; butyric acid, Sigma Chemical Co., St. Louis, Mo.; [^{3}H]thymidine, 60 Ci/mmol, Schwarz/Mann, Orangeburg, N. Y.

Results

2 mM butyrate inhibits the induction of DNA synthesis in mouse spleen lymphocytes by PHA, Concanavalin A, or endotoxin (Table I A). Although incorporation of [^{3}H]thymidine is inhibited by at least 90%, cell viability, as determined by trypan blue exclusion, is not affected. With lower concentrations

* Supported by Grants CA 16890 from the United States Public Health Service and NP-36L from the American Cancer Society.

TABLE I
Effect of Exposure to Butyrate on DNA Synthesis by Mitogen-Stimulated Lymphocytes

Butyric acid concentration (mM)	Incorporation of [³H]thymidine		
	PHA	LPS	Con A
A. Additions present throughout incubation			
0	27,300	46,400	25,229
0.1	26,900	47,050	21,590
0.5	11,740	28,990	6,302
1	6,500	11,136	1,423
2	2,184	3,248	438
B. Unbound mitogen and butyrate removed at 4 h			
0	27,100	–	–
2	26,750	–	–

Mouse spleen lymphocytes were suspended in RPMI-1640 supplemented with 10% fetal bovine serum, containing the indicated concentrations of butyric acid and either 1 μg/ml PHA, 25 μg/ml LPS, or 7 μg/ml Con A. For part B cells were washed at 4 h and resuspended in fresh medium lacking butyrate and mitogen. After a total of 38 h incubation at 37°C, 1 μCi/ml [³H]thymidine (60 Ci/mM) was added. The incubation was terminated at 40 h and incorporation of tritium into acid-insoluble material determined as described in Materials and Methods.

of butyrate, inhibition is proportionally less effective; at a concentration of 0.1 mM no inhibition is observed.

The effect of butyrate is reversible. We find that lymphocytes exposed to butyrate for as long as 17 h in the presence of mitogen are capable of unimpaired blast formation when butyrate is removed; the incorporation of thymidine, uridine, and amino acids by these cells is comparable to that observed when freshly isolated lymphocytes are exposed to mitogens.

Our data suggest that the following parameters are unaffected:

Mitogen Binding. Lymphocytes were exposed to PHA for 4 h. At the end of this incubation, the cells were washed free of unbound mitogen. Conditions were chosen such that neither a prolongation of exposure time for binding, an increase in mitogen concentration, or a second exposure of the stimulated lymphocytes to mitogen resulted in significant additional DNA synthesis. When butyrate was added during the period of mitogen binding and removed with the unbound mitogen, DNA synthesis began at approximately the same time and continued at the same rate as in cultures which were not exposed to butyric acid (Table I B).

"Early" Reactions. With experimental conditions such that the onset of detectable DNA synthesis begins 18–20 h after the addition of PHA and continues with an increasing rate for at least 30 h, addition of butyrate for any 4-h period during the first 16 h does not alter the time of onset or the rate of DNA synthesis. This suggests that at least some of the reactions involved in the commitment of cells to mitosis take place in the presence of butyrate or, alternatively, that upon removal of this fatty acid, the cells are able to recover rapidly from inhibition of "early" reactions. In contrast, when lymphocytes are exposed to butyrate for the entire 17-h period after addition of PHA, the time interval between removal of butyrate and ensuing DNA synthesis is 17 h, as if the series of reactions leading to DNA synthesis had begun only with the removal of butyrate. However, lymphocytes incubated in growth medium for 17 h before addition of PHA show a pronounced lag in the onset of DNA synthesis

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TABLE II
Effect of Prolonged Exposure to Butyrate on DNA Synthesis by Mitogen-Stimulated Lymphocytes

Hour*		20	23	26	29	40	43	46
Hour of second incubation period		3	6	9	12	23	26	29
1st incubation (0-17 h)	2nd incubation (17 h to term)	Incorporation of [^{3}H]thymidine						
					cpm/2h			
PHA	NA	6,000	8,900	14,400	18,300	–	–	–
NA	PHA	–	–	–	–	4,000	6,300	9,100
PHA, butyrate	NA	–	400	600	–	9,000	14,800	20,000
PHA, butyrate	PHA, butyrate	360	390	350	–	200	250	–

NA, no addition.
Mouse spleen lymphocytes (1.5 × 10^6/ml) were incubated at 37°C in MEM supplemented with 10% FCS with added PHA or butyrate as indicated. [^{3}H]thymidine (1 μCi/ml) was added 2 h before the termination of incubation. All other details as in Table I.
* Time measured from start of first incubation. At hour 17, all cultures were washed and resuspended in fresh medium containing the indicated additions.

compared to either freshly isolated cells or cells incubated with the mitogen and butyrate for the same time period (Table II).

Thus, the data are compatible either with the possibility that in the presence of PHA and butyrate the sequence of reactions leading to DNA synthesis progresses normally but is offset by the prolonged incubation or that prolonged exposure to PHA and butyrate does not permit the sequence of reactions to progress, but maintains the cells in the same state of inducibility or cell cycle distribution as freshly prepared lymphocytes, a state that is altered during incubation at 37°C in the absence of mitogen.

DNA Synthesis. If butyrate is added to PHA-stimulated lymphocytes at any time before the onset of detectable DNA synthesis and is not removed from the culture, the expected synthesis is completely blocked. Once DNA synthesis has commenced, the addition of butyrate has no immediate inhibitory effect on ongoing synthesis, but rather rapidly obliterates the expected increase in the rate of DNA synthesis. Depending on the time of addition of butyrate, DNA synthesis may continue at an unaltered rate for 12-16 h. With longer exposure times, the rate of DNA synthesis declines, usually well before a drop in the rate of DNA synthesis is observed in control cultures (Fig. 1*a*). Removal of butyrate results in a rapid increase in the rate of DNA synthesis. After a short lag, this rate usually exceeds that seen in untreated cultures (Fig. 1*b*).

Discussion

When mitogen is bound to lymphocytes, major changes are initiated in the array of biochemical and physical properties characteristic of this resting cell. Although a number of these changes can be shown to occur within an hour of mitogen binding (3), mitogen must be bound for a much longer time (12-16 h) to initiate maximal DNA synthesis and cell division (4). Thus, it has proven difficult to distinguish those mitogen-stimulated reactions which are prerequisites for initiation of DNA synthesis from those which are incidental to the

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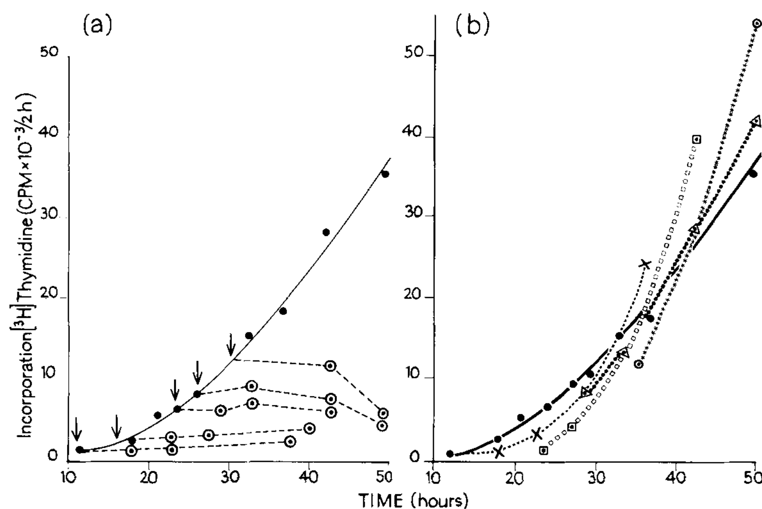

FIG. 1. Effect of butyrate on ongoing DNA synthesis. (*a*) (·—·), no butyrate; (⊙---⊙), 2 mM butyrate added at time indicated by arrow. (*b*) Butyrate added for 4-h period. (·—·), no butyrate; (---), butyrate (2 mM) present from (×) 12-16 h; (⊡) 17-21 h; (△) 23-27h; (⊙) 27-31 h. Mouse spleen lymphocytes (1.5 × 10^6) were incubated at 37°C in MEM supplemented with 10% FCS. 1 μg/ml PHA was added to all culture tubes at 0 h. Butyric acid (2 mM) was added as indicated. Time points indicated are at the termination of 2-h labeling period. All other details as in Table II.

process. Although our studies are preliminary and our conclusions are based entirely on the effect of butyrate on the kinetics of blast formation monitored through DNA synthesis, we feel that our results indicate the potential of butyrate for this purpose.

Among its useful characteristics are:

Low Toxocity. Lymphocytes and a large variety of other mammalian cells remain viable for 60 h or more in the presence of concentrations of butyrate sufficient to inhibit blast formation.

Reversibility. Our observations with mouse lymphocytes (Fig. 1), a number of mouse cell lines, and several long-term human lymphoid lines indicate that all detectable effects of butyrate on DNA synthesis, cell division, and cell morphology can be reversed in a relatively short time by simply removing the compound from the growth medium.

Rapid Uptake. Butyrate quickly reaches effective intracellular concentrations as evidenced both by its immediate inhibitory effect on the rate of increase in DNA synthesis (Fig. 1) and by its incorporation into cellular lipids and cholesterol. Incorporation of ^{14}C from carboxy-labeled butyric acid into the cholesterol and lipids of PHA-stimulated lymphocytes is on the order of 0.5 nmol/10^6 cells per h. Butyrate, as a normal metabolic intermediate, also quickly inhibits incorporation of $^{14}COOH\text{-}CH_3$ into lipids.

Lack of Effect on Mitogen Binding (Table I B)

Apparent Lack of Effect on Early Reactions

Lack of Effect on Ongoing DNA Synthesis. Butyrate permits a continuation of, but not an increase in, the rate of ongoing DNA synthesis in stimulated

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lymphocytes, suggesting that this compound interferes either with reaction(s) directly involved in or necessary for the initiation of new DNA synthesis.

It should be noted that the same effects of butyrate on the rate of DNA synthesis are observed with endotoxin-stimulated cells (B lymphocytes) and with synchronized cultures of long-term lymphoid lines or mouse L cells. Thus, an exploration of the effect of butyrate on the metabolic state of induced lymphocytes may yield information pertinent not only to the process of blast formation but to the initiation of DNA synthesis in eukaryotic cells in general.

Received for publication 2 August 1976.

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