Biochimica et Biophysica Acta, 529 (1978) 38—43
© Elsevier/North-Holland Biomedical Press

BBA 57164

THE EFFECTS OF DIBUTYRYL CYCLIC ADENOSINE 3′:5′-MONOPHOSPHATE ON CONCANAVALIN A-STIMULATED STEROL AND FATTY ACID SYNTHESIS IN MOUSE SPLEEN LYMPHOCYTES

JAMES G. RESPESS [a], JOHN D. STUBBS [a] and DONALD A. CHAMBERS [b]*

[a] *Department of Cell and Molecular Biology, San Francisco State University, San Francisco, Calif. 94132 and* [b] *Departments of Dermatology and Biological Chemistry, University of Michigan Medical School, Ann Arbor, Mich. 48109 (U.S.A.)*

(Received October 10th, 1977)

Summary

Substantial increases in both 3β-OH sterol and fatty acid synthesis were observed after concanavalin A addition to mouse spleen lymphocytes cultured in serum-free media. The rate of sterol synthesis increased linearly up to 60 h. The rate of fatty acid synthesis increased up to 20 h, reaching a plateau in synthetic activity which was the maintained. CO_2 production from acetate was slightly stimulated by concanavalin A. In contrast to sterol and fatty acid synthesis, the rate of CO_2 production in both mitogen-stimulated and resting cultures declined with time. Dibutyryl cyclic AMP had a strong inhibitory effect on concanavalin A-stimulated sterol and fatty acid synthesis from acetate, but only a slight effect on CO_2 production. Delayed addition of dibutyryl cyclic AMP resulted in reduced inhibition. The data suggest a sequence of initiation for fatty acid and sterol synthesis prior to DNA synthesis and a possible regulatory role of cyclic AMP in this initiation. The results support the hypothesis that lymphocyte activation is sequential within the spleen cell population and is accompanied by fatty acid and sterol synthesis.

Introduction

Mouse spleen lymphocytes in serum-free culture are stimulated by the plant lectin concanavalin A to increase the rate of DNA synthesis by as much as 1000-fold over the rate in resting cultures [1,2]. Lectin stimulation of lymphocytes may be analogous to immunological activation [3]. The ability to activate lymphocytes in serum-free culture provides an excellent system for studying G_0-G_1-S transitions of the cell cycle [4].

* To whom correspondence should be sent.

Previous observations in this and other laboratories have shown that cyclic AMP and its permeable derivatives are inhibitory to lymphocyte activation [1,2,4,5]. The work described in this communication implicates the involvement of 3β-OH sterols and fatty acids in lymphocyte activation and suggests that cyclic AMP may exert metabolic regulation on pathways of acetate metabolism during lymphocyte activation.

Materials and Methods

Spleen cells from C3H-F mice were prepared as described by Chambers et al. [1]. Freshly dissected spleen cells were washed three times in phosphate-buffered saline and then suspended in serum-free RPMI 1640 (obtained from the University of California Cell Culture Facility, San Francisco) containing antibiotics, at an initial cell density of $5 \cdot 10^6$ cells/ml. The lymphocyte cultures were incubated at 37°C in a humidified 5% CO_2/95% air atmosphere. An initial decline in cell number was observed during the first 10—20 h in the serum-free cultures but no further change in viability, as determined by the ability of cells to exclude Trypan blue, was seen during the course of the experiment. Cell viability remained constant at approx. $3 \cdot 10^6$ cells/ml. Cultures were activated with concanavalin A (0.5 μg/ml from Miles-Yeda, Elkhart, Ind.). This concentration of concanavalin A was found to be maximally stimulatory as reported previously [1]. Concentrations of concanavalin A greater than 1 μg/ml in serum-free culture resulted in increased cellular toxicity. Dibutyryl cyclic AMP (obtained from the Sigma Chemical Co., St. Louis, Mo.) was maximally inhibitory at concentrations greater than $2 \cdot 10^{-4}$ M, and was routinely used at $5 \cdot 10^{-4}$ M. This concentration of cyclic nucleotide has no effect on cell viability. The rate of [^{3}H]thymidine incorporation into DNA was measured by precipitating sonicated lymphocyte lysates with 10% trichloroacetic acid as previously described [1]. 2.5 μCi [^{3}H]thymidine (specific activity 13 Ci/mmol, Schwartz-Mann, Orangeburg, N.Y.) per ml of culture was added 2 h before termination of the experiment. The rates of 3β-OH sterol synthesis (assayed as digitonin-precipitable sterol), fatty acid synthesis and CO_2 evolution were measured after addition of 5 μCi/ml of culture of [1-^{14}C]acetate (specific activity 58.3 Ci/mol, New England Nuclear, Boston, Mass.) at a concentration of $8.6 \cdot 10^{-5}$ M for 4 h by the method of Kandutsch and Saucier [6]. Each data point represents the mean value of triplicate cultures, and each experiment was repeated at least three times.

Results

The temporal study of [^{3}H]thymidine incorporation into DNA (Fig. 1) showed that DNA synthesis began to increase about 20 h after concanavalin A addition. This finding is in agreement with observations by Loeb et al. [7]. The rate of synthesis continued to increase for at least 65 h, at which point the experiment was terminated. When dibutyryl cyclic AMP ($5 \cdot 10^{-4}$ M) was added with concanavalin A, the rate of [^{3}H]thymidine incorporation was greatly reduced (90% at 45 h after a 2 h pulse). Neither resting cultures nor resting cultures containing dibutyryl cyclic AMP showed any change in the rate of [^{3}H]-

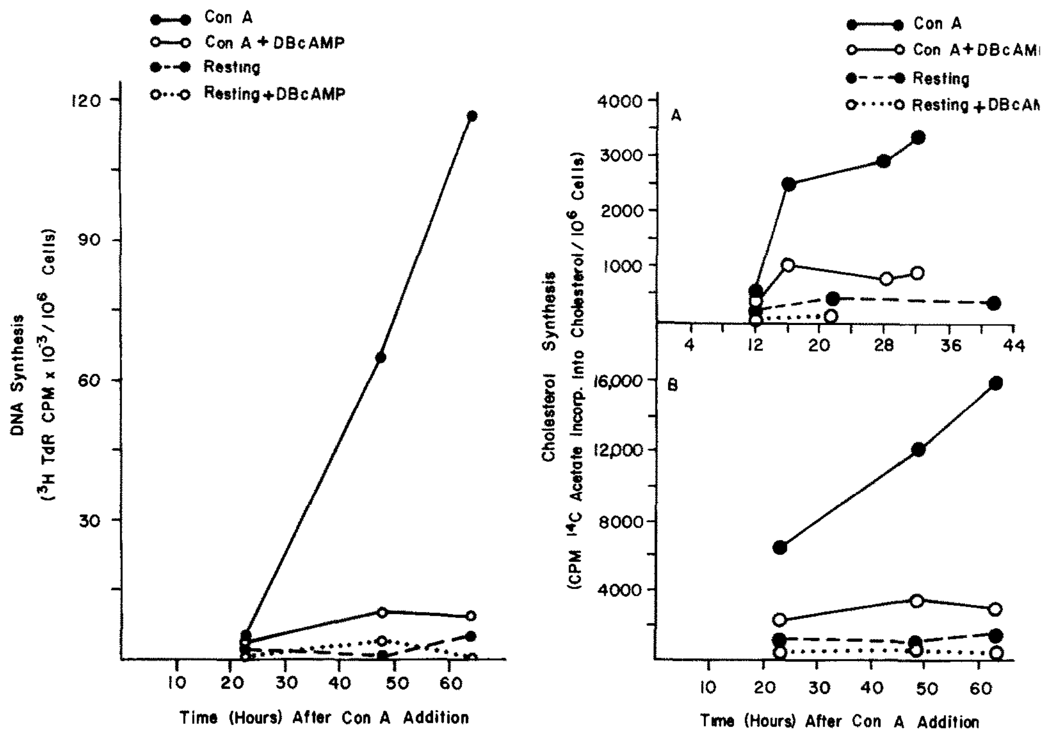

Fig. 1. The effect of concanavalin A stimulation on DNA synthesis. Cells were treated as described in Materials and Methods. $5 \cdot 10^6$ lymphocytes/ml were incubated in the presence of 0.5 μg concanavalin A (Con A) and 0.5 mM dibutyryl cyclic AMP (DBc AMP)/ml of culture, which were added to the culture media at the beginning of the experiment. DNA synthesis was measured by processing precipitated DNA after pulsing the cultures with [^{3}H]thymidine as described in Materials and Methods.

Fig. 2. (A and B) The effect of concanavalin A stimulation on cholesterol synthesis. Cells were treated as described in Fig. 1 and Materials and Methods. Concanavalin A (Con A) and dibutyryl cyclic AMP (DBc AMP) were added at the beginning of the experiments. Cholesterol was assayed as described in Materials and Methods.

thymidine incorporation during the course of the experiment. 5'-Adenosine monophosphate or sodium butyrate, added at equivalent concentrations did not effect the [^{3}H]thymidine incorporation of resting cells or concanavalin A-activated cells.

Parallel experiments designed to demonstrate the rates of [^{14}C]acetate incorporation into sterols, fatty acids and CO_2 were performed. Concanavalin A addition to lymphocyte cultures resulted in enhanced sterols synthesis and this enhancement occurred prior to concanavalin A-induced DNA synthesis (Fig. 2A). The rate of sterol synthesis continued to increase in a linear fashion for at least 65 h (Fig. 2B). Dibutyryl cyclic AMP added concomitantly with concanavalin A resulted in a reduction in the rate of [^{14}C]acetate incorporation into sterol. The extent of inhibition at 45 h was 84%. The rate of [^{14}C]acetate incorporation into sterol did not change for resting cultures containing dibutyryl cyclic AMP (Figs. 2A and 2B). The results shown in Fig. 2A indicate that enhanced sterol synthesis began at 8—12 h following concanavalin A addition.

Figs. 3A and 3B show the incorporation of [^{14}C]acetate into saponifiable

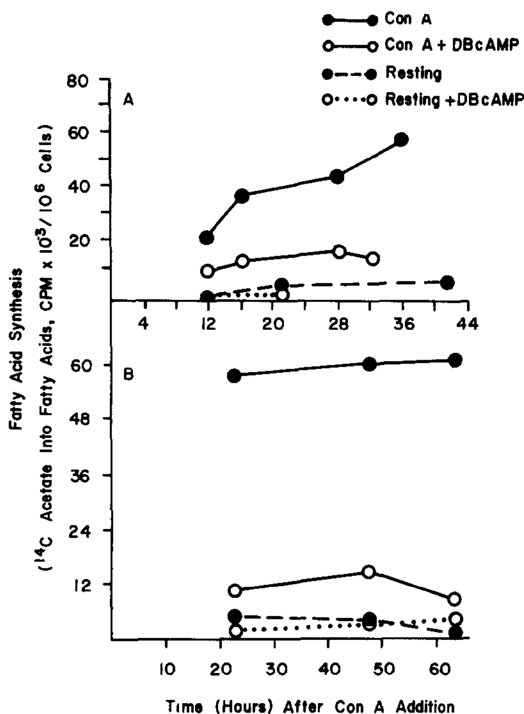

Fig. 3. (A and B) The effect of concanavalin A stimulation on fatty acid synthesis. Cells were treated as in Fig. 2, and [^{14}C]acetate incorporation into fatty acids was assayed as decribed in Materials and Methods.

fatty acids after the addition of concanavalin A to lymphocyte cultures. By 22 h, the rate of concanavalin A-stimulated fatty acids synthesis was 20-fold greater than the resting rate and was maintained at this elevated rate up to 65 h. Dibutyryl cyclic AMP inhibited the incorporation of [^{14}C]acetate into concanavalin A-stimulated fatty acids by 88% at 45 h. A comparison of Figs. 2A and 3A show that this stimulation of acetate incorporation into fatty acids began prior to the increase in sterol synthesis.

Concanavalin A stimulated the production of $^{14}CO_2$ from [^{14}C]acetate only 2-fold (Fig. 4). However, CO_2 production gradually decreased under all experimental conditions and the cause of this decrease is not clear. The situation could be explained by a decrease in the rate of metabolism of acetate through the tricarboxylic acid cycle, in cells maintained in serum-free media. If this hypothesis is valid, the fall in CO_2 production is apparently independent of concanavalin A-mediated effects on fatty acid and sterol biosynthesis.

In order to provide further insight into the nature of the cyclic AMP response, we have examined the temporal relationship of dibutyryl cyclic AMP addition to concanavalin A-stimulated lymphocytes with respect to fatty acid synthesis, sterol synthesis and DNA synthesis. The observations noted above, suggested a sequential order for cyclic AMP effects on these metabolic processes. The data of Fig. 5 demonstrate the effects of delayed addition of dibutyryl cyclic AMP on concanavalin A-stimulated DNA, fatty acid and sterol synthesis. A study of DNA synthesis revealed that when dibutyryl cyclic AMP was added concomi-

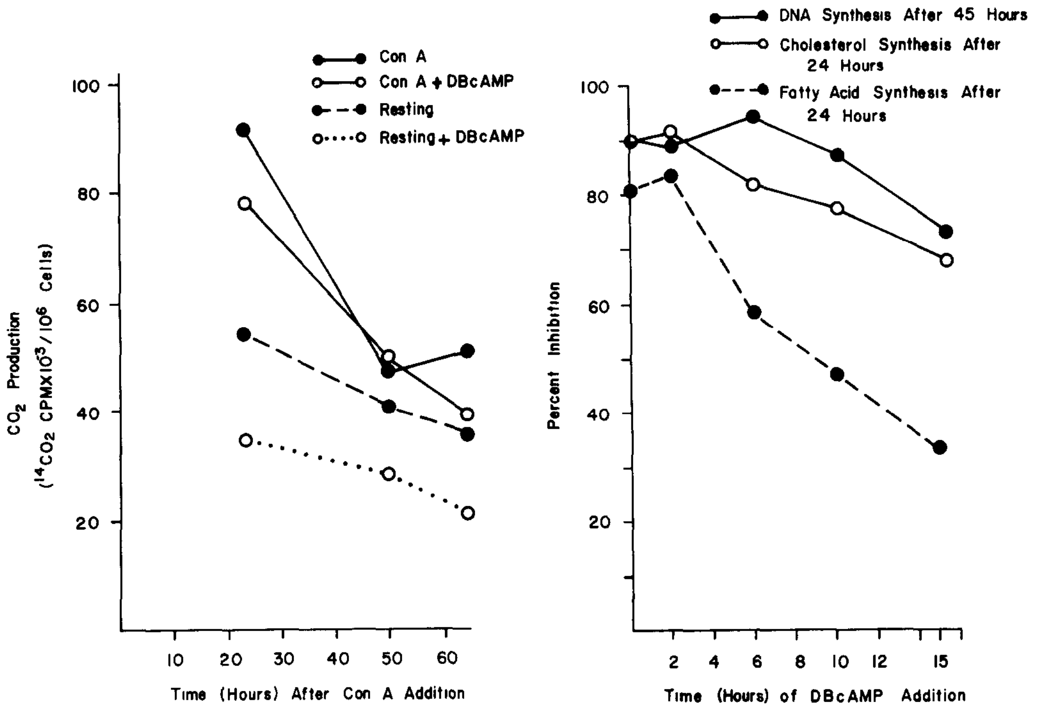

Fig. 4. The effect of concanavalin A stimulation on CO_2 production. Cells were treated as in Fig. 2, and $^{14}CO_2$ production was assayed as described in Materials and Methods.

Fig. 5. The effects of delayed addition of dibutyryl cyclic AMP on concanavalin A stimulation. Cells were treated as described in Fig. 1, except that 0.5 mM dibutyryl cyclic AMP (DBc AMP) was added at the indicated time points after the addition of concanavalin A (Con A). DNA synthesis, cholesterol synthesis and fatty acid syntheis were assayed as described in Materials and Methods.

tantly with concanavalin A, a maximal inhibition of 90% occurred, as observed in previous work with Balb/C mice [1]. When dibutyryl cyclic AMP was added 2–6 h after concanavalin A, maximal inhibition was still observed. However, additions of dibutyryl cyclic AMP after 6 h resulted in decreased inhibition with time. Similar effects were observed for fatty acid and sterol synthesis, but in these cases maximum inhibition occurred only when dibutyryl cyclic AMP was added at 2 h or before. After 2 h, the inhibitory effects declined gradually as a function of the time of dibutyryl cyclic AMP addition.

The observed effects of delayed addition of dibutyryl cyclic AMP on mitogen-induced DNA synthesis are in agreement with the report of Smith et al. [8], who concluded that dibutyryl cyclic AMP acts at an early phase of lymphocyte transformation; this also compliments previous observations in this laboratory [4]. Our data demonstrate that dibutyryl cyclic AMP inhibits sterol and fatty acid synthesis early in the G_1 phase of lymphocyte transformation.

Discussion

The data presented in this study show that concanavalin A activation of mouse spleen lymphocytes is accompanied by a pronounced increase in both

3β-OH sterol and fatty acid synthesis. Sterol synthesis increases at least 8 h before DNA synthesis. Fatty acid synthesis increases before sterol synthesis. Dibutyryl cyclic AMP inhibits the biosynthesis of all three of these products.

The decreasing effect of dibutyryl cyclic AMP when added at times after concanavalin A supports the hypothesis by Gunther et al. [9] that the cells, or subpopulation of cells, are sequentially committed to activation in the presence of lectin.

An argument consistent with the data presented above is that dibutyryl cyclic AMP inhibition of DNA synthesis is not tightly coupled to parallel regulation of fatty acid and sterol biosynthesis but that DNA synthesis has a requirement for enhanced fatty acid and/or sterol biosynthesis. Alternatively, dibutyryl cyclic AMP may regulate different events in the proliferative program in parallel. Nevertheless, the hypothesis by Chen et al. [10] that increased sterol synthesis may be a prerequisite for DNA synthesis in mitogen-stimulated lymphocytes is supported by this data. The possibility exists that the inhibitory effect of dibutyryl cyclic AMP on DNA synthesis may relate to prior inhibition of sterol or fatty acid synthesis, and that the synthesis of sterol or its precursors may be important in the regulation of the cell's proliferative program.

Acknowledgements

We would like to thank Jeanne Emberling for her assistance during the course of this work. We also thank Dr. John Watson for his helpful advice and discussion. This work was supported by grants from the National Institute for Dental Research, N.I.H. No. 1R01 DE 04313 (D.A.C.) and the National Institute for Arthritis, Metabolic and Digestive Diseases, N.I.H. No. 2P01 AM 15740 (D.A.C.).

References

1 Chambers, D.A., Martin, D.W. and Weinstein, Y. (1974) Cell 3, 375—380
2 Weinstein, Y., Chambers, D.A., Bourne, H.R. and Melmon, K.L. (1974) Nature 251, 352
3 Greaves, M.F. and Janossy, G. (1972) Transplant Rev. 11, 37—130
4 Chambers, D.A. (1976) J. Invest. Dermatol 67, 661—664
5 Braun, W., Lichtenstein, L.M. and Parker, C.W. (1974) Cyclic AMP, Cell Growth, and the Immune Response, Springer-Verlag, New York
6 Kandutsch, A.A. and Saucier, J.E. (1969) J. Biol. Chem. 244, 2299-2305
7 Loeb, L.A., Ewald, J.L. and Agarwal, S.S. (1970) Cancer Res. 30, 2514—2520
8 Smith, J.W., Steiner, A.L. and Parker, C.W. (1971) J. Clin. Invest. 50, 442—448
9 Gunther, G.R., Wand, J.L. and Edelman, G.M. (1974) J. Cell Biol. 62, 366—377
10 Chen, H.W., Heiniger, H. and Kandutsch, A.A. (1975) Proc. Natl. Acad. Sci. U.S. 72, 1950—1954