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Control of Growth of a Tumor Cell by Linoleic Acid

(cell cycle/control of DNA synthesis/serum lipids/unsaturated fatty acids)

ROBERT W. HOLLEY, JULIA H. BALDWIN, AND JOSEPHINE A. KIERNAN

The Salk Institute, Post Office Box 1809, San Diego, California 92112

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ABSTRACT **The growth of mouse myeloma XS 63·5 cells in cell culture is dependent on serum. Among the several growth factors present in serum, the lipid fraction is highly active. The growth factor(s) provided by the serum lipid fraction can be replaced by unsaturated fatty acids. If XS 63·5 cells are cultured in medium that is low in serum lipids, the cells become quiescent, with most of the cells in the G_1 (or G_0) phase of the cell cycle. Addition of linoleic acid to such quiescent cells leads to reinitiation of DNA synthesis and growth.**

As part of a study of culture conditions that arrest the growth of tumor cells in the G_1 (or G_0) phase of the cell cycle, we have investigated the factors that control the growth of the XS 63·5 cell, a mineral oil-induced mouse myeloma cell (1). Our initial finding was that the growth of these cells can be arrested by using the culture conditions developed by Glinos and Weerlein (2) for the arrest of growth of L929 cells. Their conditions involve daily centrifugation of the cell culture and resuspension of the cells in fresh medium. Under these conditions, the cell density attained by XS 63·5 cells is dependent on the concentration of serum in the medium. Among the several factors present in serum, the serum lipid fraction is especially active. The present paper is concerned with the response of XS 63·5 cells to serum lipids and to unsaturated fatty acids.

MATERIALS AND METHODS

Cell Culture. XS 63·5 cells (1) were obtained originally from Dr. M. Cohn. The cells used in these experiments had recently been passed, as an ascites tumor (3), through a Balb/c mouse. Stock cells were cultured in suspension, in plastic petri dishes, in Dulbecco–Vogt modified Eagle's medium (4) with 10% (v/v) horse serum at 37° in a CO_2 incubator.

Assay of Serum Fractions, Serum Lipids, and Fatty Acids. XS 63·5 cells in late logarithmic phase of growth were collected by centrifugation and were resuspended at 2×10^5 cells per ml in Dulbecco–Vogt modified Eagle's medium with (Fig. 1 and Table 1) 1% horse serum or (Fig. 3 and Table 2) 2% (v/v) of a solution of lipid-free serum proteins [fraction A of Kaplan and Bartholomew (5)]. Five-milliliter aliquots of the cell suspension were added to 5.5-cm plastic petri dishes. Solutions of the materials to be assayed were added to the dishes immediately. Lipids and fatty acids were added as ethanol solutions (2 μl of ethanol per ml of medium) or as autoclaved solutions in Tween acetate (6) (20 μl of a 1% Tween acetate solution per ml of medium). (Controls indicated that ethanol and Tween acetate at these concentrations neither inhibited nor stimulated growth of XS 63·5 cells.) The cell densities were determined after 3 or 4 days, with a Coulter Counter.

Flow Microfluorometric Analyses. The procedure was that of Tobey *et al.* (7). The cells were collected by centrifugation and were washed twice by resuspension and centrifugation in calcium- and magnesium-free phosphate-buffered saline. The washed cells were fixed for 2 hr to 3 days in the cold (4°) with a 3.7% formaldehyde solution in saline, and the cells were stained with acriflavin. The cells were analyzed in a Los Alamos design microfluorometer (8) with an argon laser at 488 nm. Patterns obtained from the storage oscilloscope are shown in Figs. 2 and 4.

RESULTS AND DISCUSSION

Reversible Arrest of Growth of XS 63·5 Cells. XS 63·5 cells, cultured under the conditions of Glinos and Weerlein (2), with daily centrifugation and resuspension of the cells in fresh medium, reach a constant cell density after a number of days of culture. With 10% horse serum in Dulbecco–Vogt modified Eagle's medium, the final cell density is about 2.5×10^6 cells per ml. With 2% horse serum, the final cell density is lower, about 6×10^5 cells per ml, indicating that the cell density attained is determined by the concentration of serum in the medium. If the cells are resuspended in fresh medium at a lower cell density, growth is reinitiated.

These results suggested that reversible arrest of growth of XS 63·5 cells might be demonstrated without the daily medium changes if the cells were grown in medium with a low

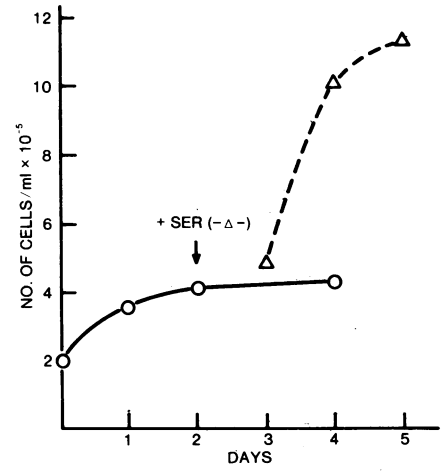

FIG. 1. Growth curves of XS 63·5 cells in Dulbecco–Vogt modified Eagle's medium with 1% horse serum (○), and after the serum concentration was raised to 10% in some of the dishes on day 2 (△).

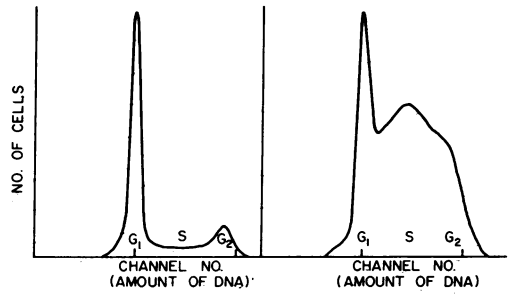

FIG. 2. Flow microfluorometric analyses showing DNA contents per cell in (left) a population of quiescent XS 63·5 cells obtained at day 2 of Fig. 1, and (right) 17 hr after the addition of serum to the quiescent cells. The percentage of cells in S phase has increased from about 20% to 75%.

serum concentration. At a low serum concentration, the final cell density attained should be low and medium constituents other than serum should suffice for several days of experimentation. Results under such conditions are shown in Fig. 1. In medium with 1% horse serum, growth of XS 63·5 cells ceases after 2 days, at about 4×10^5 cells per ml. Growth resumes when serum is added to the medium.

Flow Microfluorometric Analyses of XS 63·5 cells under the culture conditions of Fig. 1 are shown in Fig. 2. Quiescent XS 63·5 cells, obtained by culture of the cells in medium with 1% serum, are found to be primarily in the G_1 (or G_0) phase of the cell cycle. Addition of serum to these quiescent cells leads to the reinitiation of DNA synthesis, at about 14 hr, and by 17 hr most of the cells are in the S phase of the cell cycle (Fig. 2). Thus, it appears that these tumor cells have a "normal" G_1 (or G_0) growth arrest mechanism, with growth in cell culture controlled by serum factors.

Activity of Serum Lipids. Assays of fractions of serum for their growth-stimulating activity in XS 63·5 cell cultures established that serum contains several growth factors (Table 1). These results are similar to the results obtained with other mammalian cells (5). In contrast to the results with other

TABLE 1. *Growth of XS 63·5 cells in response to serum fractions*

Additions	No. of cells per ml $\times 10^{-5}$
None (1% horse serum)	4.6
1% Mouse serum (0.5 mg of protein/ml)	14
4% Fraction A: lipid-free mouse serum proteins (0.2 mg/ml*)	6.6
4% Fraction B: aqueous methanol-soluble fraction of mouse serum (1 mg/ml*)	9
2% Fraction C: lipid fraction of mouse serum (0.1 mg/ml*)	11
Fractions A + B	11
Fractions A + C	12
Fractions B + C	10
Fractions A + B + C	12

* The amounts of fractions A, B, and C (prepared according to ref. 5) were chosen to give the maximum growth responses obtainable with the individual fractions. For conditions of the assay, see *Materials and Methods.*

TABLE 2. *Growth of XS 63·5 cells in response to fatty acids and serum lipids*

Additions	No. of cells per ml $\times 10^{-5}$
None (2% fraction A)	3.1
Lipid fraction of mouse serum (0.1 mg/ml)	7.6
Myristic acid (5 μg/ml)	4.0
Palmitic acid (5 μg/ml)	3.7
Stearic acid (5 μg/ml)	3.2
Elaidic acid (5 μg/ml)	4.3
Oleic acid (5 μg/ml)	7.4
Linoleic acid (5 μg/ml)	7.7
Arachadonic acid (5 μg/ml)	6.4
Mouse serum (1%)	11.0

For conditions, see *Materials and Methods.*

cells, the lipid fraction is the most active for growth of XS 63·5 cells.

Fractions of the mouse serum lipids were then assayed for growth activity with medium prepared with lipid-free serum proteins instead of with whole serum. Mouse serum lipids were fractionated according to Rouser *et al.* (9), and the active lipid was found in the neutral lipid fraction. Further fractionation of the neutral lipids on silicic acid according to Barron and Hanahan (10) indicated that the triglyceride fraction was most active. This result suggested the possibility that fatty acids might be active. As shown in Table 2, oleic, linoleic, and arachadonic acids stimulate growth of XS 63·5 cells. Oleic and linoleic acids are as active as the mouse serum lipid fraction, but are not as active as whole serum. Mouse serum lipids were found to contain about 7% linoleic acid, by the procedure of Holman (11), indicating that linoleic acid can account for the activity of the lipid fraction. Prostaglandins E_1, E_2, $F_1\alpha$, and $F_2\alpha$ were inactive under the assay conditions.

Control of Growth by Linoleic Acid. Growth curves (Fig. 3) of cells cultured under the conditions used for the lipid assays indicated that the growth of XS 63·5 cells ceases a day or two after the cells are transferred into medium with lipid-free serum proteins. Growth resumes after the addition of linoleic acid (Fig. 3). Flow microfluorometric analyses (Fig. 4) show that the quiescent cells are primarily in the G_1 (or G_0) phase of the cell cycle, but that 20 hr after the addition of linoleic acid a greatly increased percentage of the cells is in the S phase. Thus, when growth of XS 63·5 cells has been arrested

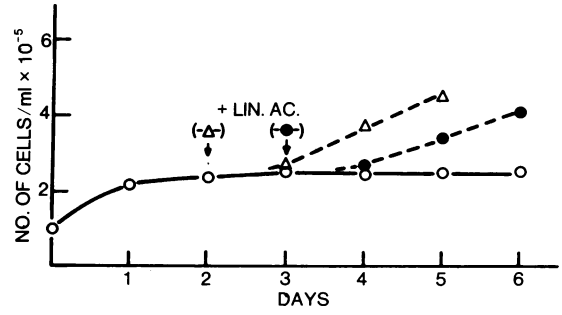

FIG. 3. Growth curves of XS 63·5 cells in Dulbecco–Vogt modified Eagle's medium with 2% of a solution of lipid-free serum proteins (○) and after the addition of linoleic acid on day 2 (△) or on day 3 (●).

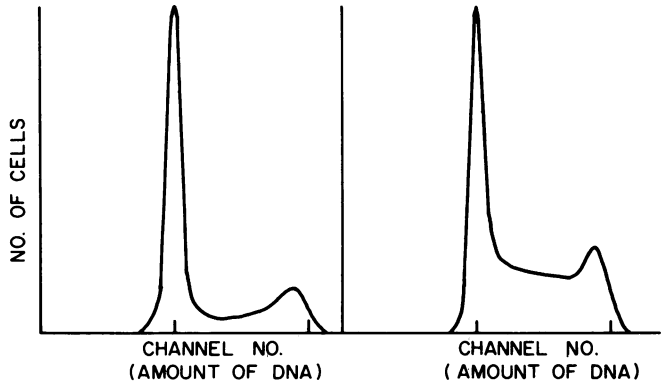

FIG. 4. Flow microfluorometric analyses showing DNA contents per cell in (left) a population of quiescent XS 63·5 cells grown in medium with lipid-free serum proteins (day 3 of Fig. 3) and (right) 20 hr after the addition of linoleic acid. The percentage of cells in S phase has increased from about 20 to 50%.

by limitation of serum lipids, the addition of linoleic acid can lead to the initiation of DNA synthesis.

The mode of action of linoleic acid in controlling the cell cycle in the XS 63·5 mouse myeloma cell is not known. There are many possibilities. The action may be direct, involving some mechanism that monitors the availability of an essential nutrient and that in turn controls the cell cycle (12), or it may be indirect, for example, affecting the fluidity of the membrane (6), or affecting the biosynthesis of some other growth-controlling material (13). Whatever the mode of action, the results indicate that unsaturated fatty acids must be added to the varied list (14) of external factors that can control DNA synthesis and growth in mammalian cells.

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