

**CHOLESTEROL REQUIREMENT OF P3-X63-Ag8 AND
X63-Ag8.653 MOUSE MYELOMA CELLS FOR GROWTH
IN VITRO**

BY J. DENRY SATO, TOMOYUKI KAWAMOTO,* AND TETSUJI OKAMOTO

*From the W. Alton Jones Cell Science Center, Lake Placid, New York 12946; and the
Department of Biochemistry, Okayama University Dental School, Okayama City 700, Japan

Since the original demonstration by Kohler and Milstein (1) of the feasibility of using cell fusion to produce antibody-secreting hybridomas, several improved mouse myeloma parent cell lines have been developed. In recent years NS-1-Ag4-1 (NS-1) (2), Sp2/0-Ag-14 (Sp2/0) (3), and X63-Ag8.653 (X63.653) (4) have been commonly used to produce hybridomas. All three of these cell lines were independently derived from the original myeloma parent line P3-X63-Ag-8 (X63) (1), and all three cell lines differ from X63 with respect to Ig gene expression. Sp2/0 cells were obtained by subcloning Sp2/3-3, an X63 hybridoma (1, 3), while NS-1 and X63.653 were subcloned in several steps from X63. An examination of the growth requirements of NS-1 cells in serum-free medium revealed that NS-1 had absolute requirement for low density lipoprotein (LDL), which was not shared by NS-1 hybridomas (5). The lipid requirement of NS-1 cells was subsequently found to be a requirement for cholesterol (6). As cholesterol auxotrophy is an exceedingly rare phenotype in cultured mammalian cells, it was of interest to determine whether NS-1 cells were unique among X63 and the other derivatives of the MOPC21 cell line P3 (7). We have found that Sp2/0 cells require neither LDL (6) nor cholesterol for growth, and here we demonstrate that X63 and X63.653 cells, like NS-1 cells, are cholesterol auxotrophs. In addition, we report that cholesterol-independent variants of X63 and X63.653 can be selected.

Materials and Methods

Maintenance of Cells and Medium Preparation. P3-X63-Ag8, X63-Ag8.653, Sp2/0-Ag14, and U937 cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in a humidified atmosphere of 5% CO₂ at 37°C in RD + 5F (factor) medium supplemented with 1% or 5% FCS (HyClone Laboratories, Logan, UT). RD + 5F medium consisted of a 1:1 mixture (by volume) of RPMI 1640 (Gibco Laboratories, Grand Island, NY) and DME (high glucose; Gibco Laboratories) with 5 µg/ml crystalline bovine insulin (Sigma Chemical Co., St. Louis, MO), 5 µg/ml human transferrin (Fe³⁺-free), 10 µM 2-ME, 10 µM 2-aminoethanol, and 10 nM sodium selenite (all from Sigma Chemical Co.). The 5F supplements were made as sterile 200× concentrates and stored at 4°C. RD medium included 15 mM Hepes buffer, 2 mM L-glutamine, 0.01% sodium pyruvate (all from Sigma Chemical Co.), 2.2 g/L sodium bicarbonate (J.T. Baker Chemical Co., Phillipsburg, NJ), 20 mg/L penicillin G (Sigma Chemical Co.), 45 mg/liter streptomycin sulfate and 40 mg/L ampicillin (both Sigma

This work was supported by National Institutes of Health grants CA-40294 and CA-37589 to G. H. Sato, and by NIH training grant AG-00095.

J. EXP. MED. © The Rockefeller University Press · 0022-1007/87/06/1761/06 \$1.00

Chemical Co.). RD medium was prepared with water purified by deionization and reverse osmosis (Milli-Q; Millipore), adjusted to pH 7.5 and sterilized by filtration through a 0.2 μm filter (Millipore Continental Water Systems, Bedford, MA).

Cell Growth Assays. The effects of several lipids on cell proliferation were examined. Cells were harvested, washed with RD medium or PBS, collected by centrifugation, and resuspended in RD + 5F medium. The cells were seeded in 24-well plates (Costar, Cambridge, MA) at 10^4 cells per well, and the following supplements were added: LDL (1.019–1.063 g/ml; Meloy Laboratories Inc., Springfield, VA); BSA–linoleic acid; cholesterol (Calbiochem-Behring Corp., La Jolla, CA) complexed with BSA (fraction V; Sigma Chemical Co.) or mevalonic acid in the form of D-L-mevalonolactone (Sigma Chemical Co.) (see figure legends). Fatty acid-free BSA (Pentax; Miles Laboratories Inc., Naperville, IL) was complexed with linoleic acid (Sigma Chemical Co.) in a 1:4 molar ratio as described previously (5). Concentration of LDL and BSA–linoleic acid are expressed in the text with respect to protein. Stock solutions of LDL (5 mg/ml), BSA–linoleic acid (50 mg/ml), mevalonolactone (100 mg/ml), and BSA (fraction V) (50 mg/ml in PBS) were stored at 4°C; cholesterol (4 mg/ml in 100% ethanol) was stored at –20°C. To prevent free cholesterol from precipitating in cell cultures the stock solutions of BSA (fraction V) and cholesterol were warmed to 37°C, and appropriate volumes of both solutions were injected individually into the medium. Cells were incubated at 37°C in an atmosphere of 5% CO_2 . The cells in duplicate cultures were counted with a model Z_{B1} counter (Coulter Electronics Inc., Hialeah, FL) on the indicated days.

Results

Fig. 1 shows the growth responses of X63 and X63.653 cells to human LDL in RD + 5F medium. Both cell lines responded to LDL and were absolutely dependent on LDL for growth. Maximum cell growth occurred in 2–6 $\mu\text{g/ml}$ LDL, and both cell lines died without proliferating in the absence of LDL.

To determine whether the LDL requirement of X63 and X63.653 cells reflected a need for cholesterol, the major lipid component of LDL (8), both cells were cultured in medium supplemented with BSA and increasing concentrations of free cholesterol (Fig. 2). Cholesterol indeed stimulated the growth of

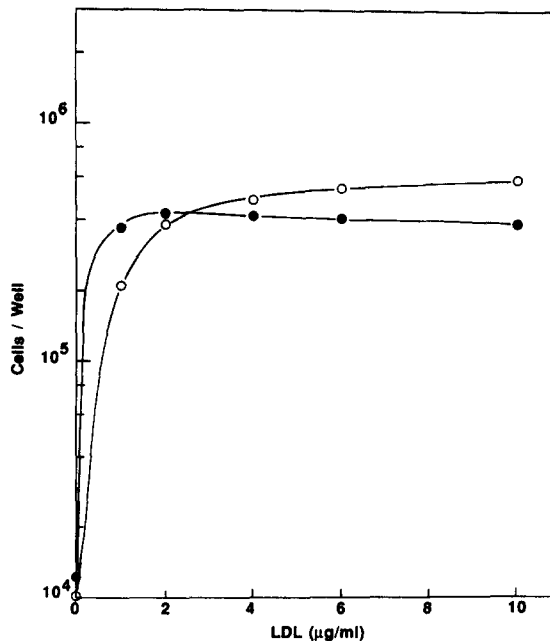


FIGURE 1. Effect of LDL concentration on the growth of X63 and X63.653 cells. X63 (●) and X63.653 (○) cells were plated at 10^4 cells per well in RD + 5F medium containing increasing concentrations of human LDL. Cells in duplicate cultures were counted after 120 h.

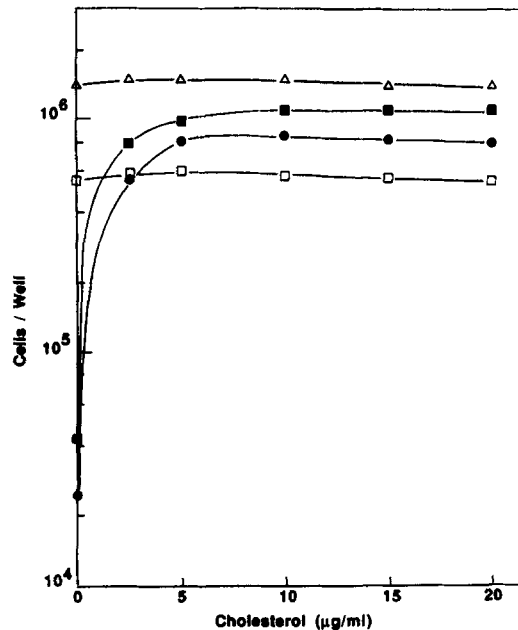


FIGURE 2. Effect of cholesterol concentration on cell growth. X63 (●), X63.653 (■), WI-L2 human B-lymphoblastoid (Δ), and U937 human histiocytic lymphoma (□) cells were plated at 10^4 cells per well in RD + 5F medium supplemented with 4 mg/ml BSA and increasing concentrations of cholesterol. Cells in duplicate cultures were counted after 120 h.

X63 and X63.653, with maximum growth occurring in 5–10 $\mu\text{g/ml}$ cholesterol. The degree of growth stimulation elicited by cholesterol was similar to that seen in optimal concentrations of LDL. BSA alone was not sufficient to support cell growth; X63 and X63.653 cells died after one and two population doublings, respectively, in the absence of exogenous cholesterol. In contrast, two human cell lines, WI-L2 B-lymphoblastoid cells (9) and U937 histiocytic lymphoma cells (10) neither required cholesterol for growth nor were stimulated by cholesterol at concentrations up to 20 $\mu\text{g/ml}$ (Fig. 2). Mevalonic acid, an intermediate in cholesterol biosynthesis, was not able to replace cholesterol in supporting the growth of X63 and X63.653, and it inhibited growth in concentrations >1 mg/ml (results not shown).

The growth kinetics of X63 and X63.653 cells in cholesterol-containing medium are shown in Fig. 3. In the presence of 10 $\mu\text{g/ml}$ cholesterol, X63 cells proliferated with a doubling time (T_d) of 17 h and reached a maximum density of 10^6 cells/ml (Fig. 3A); X63.653 cells had a T_d of 16 h and reached a density of 9×10^5 cells/ml in 6 d (Fig. 3B). Neither cell line proliferated in the absence of cholesterol, whereas cholesterol-independent Sp2/0-Ag 14 cells (6) grew with a T_d of 15 h in both the presence and absence of cholesterol (Fig. 3C).

Although the X63 and X63.653 cell lines were dependent on cholesterol or LDL for survival and growth in serum-free medium, it was possible to select cholesterol-independent variants of both after prolonged growth in low concentrations of serum. After 8 mo in RD + 5F medium supplemented with 0.5% FCS colonies of cells survived at frequencies of $1-2 \times 10^{-3}$ or less in unsupplemented RD + 5F medium (data not shown). One X63.653 subline, X63.653-PC, has been maintained in RD + 5F medium for 6 mo. X63.653-PC cells from passage 2 proliferated in RD + 5F medium with a T_d time of 28 h (Fig. 4); the rate of cell growth increased in medium supplemented with LDL ($T_d = 20$ h), LDL and BSA–linoleic acid ($T_d = 19$ h), or BSA–cholesterol ($T_d = 19$ h).

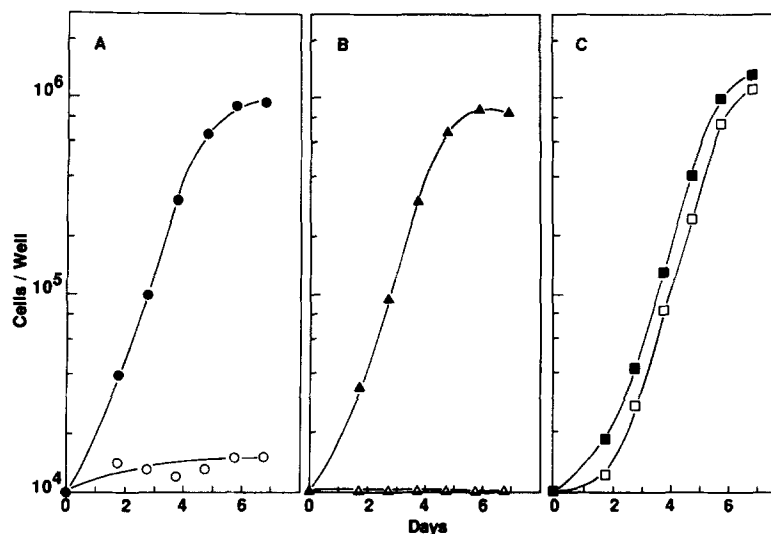


FIGURE 3. Growth kinetics of X63, X63.653, and Sp2/0 cells in the presence or absence of cholesterol. Cells were plated at 10^4 cells per well in RD + 5F medium supplemented with 4 mg/ml BSA in the presence (closed symbols) or absence (open symbols) of $10 \mu\text{g/ml}$ cholesterol. Cells in duplicate cultures were counted on the indicated days. (A) X63, (B) X63.653, and (C) Sp2/0.

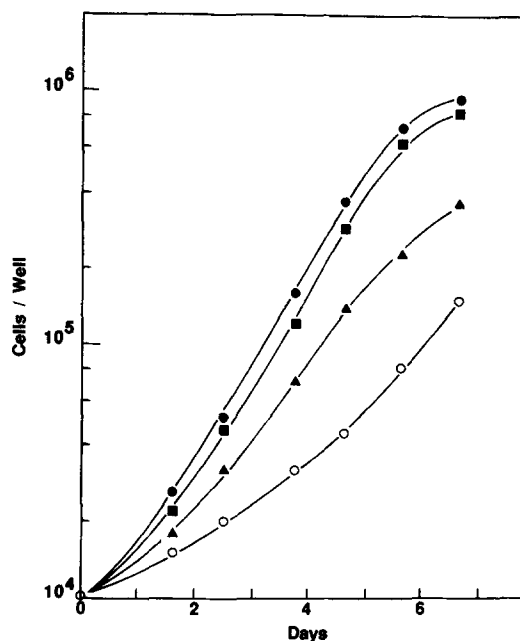


FIGURE 4. Growth of X63.653-PC cells in serum-free medium. Cholesterol-independent X63.653-PC cells (2nd passage) were plated at 10^4 cells per well in unsupplemented RD + 5F medium (○) or in RD + 5F supplemented with $10 \mu\text{g/ml}$ human LDL (▲), $10 \mu\text{g/ml}$ LDL and 0.5 mg/ml BSA-linoleic acid (■), or 4 mg/ml BSA and $10 \mu\text{g/ml}$ cholesterol (●). Cells in duplicate wells were counted on the indicated days.

Discussion

Cholesterol auxotrophy is a very rare phenotypic trait in mammalian cells. Most animal cells, including normal human (11) and mouse (12) lymphocytes, are capable of *de novo* cholesterol synthesis from acetate even though LDL in plasma and serum is the major source of cholesterol for cells in vivo and in vitro (8, 13). We have previously demonstrated that NS-1 mouse myeloma cells

required LDL or cholesterol for growth in serum-free medium (5, 6), yet the propagation of numerous other established cell lines under serum-free conditions (14–16) has revealed no additional cholesterol auxotrophs. U937 human histiocytic lymphoma cells were reported to require cholesterol for growth in vitro (17); however, we have been unable to confirm that finding (Fig. 2).

In this study we have determined that NS-1 cells are not unique among the derivatives of the MOPC21 cell line P3 (7) in their requirement for cholesterol. Both X63 and X63.653 were unable to proliferate in serum-free medium without an exogenous source of cholesterol; LDL at 2–6 $\mu\text{g/ml}$ or BSA-bound cholesterol at 5–10 $\mu\text{g/ml}$ were essential for the optimal growth of both cell lines. By contrast, Sp2/0 cells required neither LDL (6) nor BSA-cholesterol (Fig. 3C) for growth. These results indicate that the clonal derivatives of P3 cells are all cholesterol auxotrophs, whereas hybridomas produced with these cells, such as Sp2/0 and NS-1 hybridomas (5), acquire the ability to synthesize cholesterol from their normal B cell parent. As NS-1, X63, and X63.653 cells could not be rescued by mevalonic acid in cholesterol-free medium, it is evident that all three cells are defective in cholesterol biosynthesis at a point beyond the formation of mevalonic acid by 3-hydroxy-3-methylglutaryl (HMG)-coenzyme A reductase (8). Chen, et al. (18) have provided evidence that NS-1 cells are unable to convert lanosterol to cholesterol. Further investigation has suggested that NS-1, X63, and X63.653 cells can partially demethylate lanosterol and accumulate a C-28 cholesterol intermediate (Welsh, C. J., et al., unpublished results).

It is likely that the cholesterol requirements of NS-1, X63, and X63.653 cells did not arise independently but rather represent a heritable trait that has been maintained in these cells at least since the isolation of the X63 clone. Furthermore, this characteristic cannot have resulted from chromosome loss, as cholesterol-independent variants of each cell line can be selected after prolonged growth in low concentrations of serum (reference 5; this manuscript). The origin of this phenotype is unknown; however, it is plausible that cholesterol-dependent P3 or X63 cells were selected by exposure to polyene fungicides commonly used in cell culture. The toxic effects on cells of amphotericin B and filipin are mediated by the binding of these antibiotics to membrane sterols (19), and this mechanism of action of these agents has been exploited to select cholesterol-deficient mutants of mouse LM fibroblasts (20) and Chinese hamster ovary cells (21). This possibility serves to emphasize that in vitro culture conditions may exert on cells selection pressures that are neither intended nor anticipated.

Summary

P3-X63-Ag8 and X63-Ag8.653 mouse myeloma cells have an absolute requirement for cholesterol for growth under serum-free conditions. This requirement can be satisfied by low density lipoprotein at 2–6 $\mu\text{g/ml}$ or by BSA-bound cholesterol at 5–10 $\mu\text{g/ml}$. Cholesterol-independent variants can be selected after prolonged growth in low concentrations of serum.

We thank Julie Lamb and Carla Cheyne for secretarial assistance and Marina LaDuke and Takako Hoshi for graphic art work. Supported by NIH grants CA 40294 and CA 37589 to G. H. Sato and AG 00095.

Received for publication 17 February 1987.

References

1. Kohler, G., and C. Milstein. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature (Lond.)* 256:495.
2. Kohler, G., S. C. Howe, and C. Milstein. 1976. Fusion between immunoglobulin-secreting and nonsecreting myeloma cell lines. *Eur. J. Immunol.* 6:292.
3. Schulman, M., C. D. Wilde, and G. Kohler. 1978. A better cell line for making hybridomas secreting specific antibodies. *Nature (Lond.)* 276:269.
4. Kearney, J. F., A. Radbruch, B. Liesgang, and K. Rajewsky. 1979. A new mouse myeloma cell line that has lost immunoglobulin expression but permits the construction of antibody-secreting hybrid cell lines. *J. Immunol.* 123:1548.
5. Kawamoto, T., J. D. Sato, A. Le, D. B. McClure, and G. H. Sato. 1983. Development of a serum-free medium for growth of NS-1 mouse myeloma cells and its application to the isolation of NS-1 hybridomas. *Anal. Biochem.* 130:445.
6. Sato, J. D., T. Kawamoto, D. B. McClure, and G. H. Sato. 1984. Cholesterol requirement of NS-1 mouse myeloma cells for growth in serum-free medium. *Mol. Biol. & Med.* 2:121.
7. Horibata, K., and A. W. Harris. 1970. Mouse myelomas and lymphomas in culture. *Exp. Cell Res.* 60:61.
8. Goldstein, J. L., and M. S. Brown. 1977. The low-density lipoprotein pathway and its relation to atherosclerosis. *Annu. Rev. Biochem.* 46:897.
9. Levy, J. A., M. Virolainen, and V. Defendi. 1968. Human lymphoblastoid lines from lymph node and spleen. *Cancer (Phila.)* 22:517.
10. Sundström, C., and K. Nilsson. 1976. Establishment and characterization of a human histiocytic lymphoma cell line (U-937). *Int. J. Cancer.* 17:565.
11. Ho, Y. K., J. R. Faust, D. W. Bilheimer, M. S. Brown, and J. L. Goldstein. 1977. Regulation of cholesterol synthesis by low density lipoprotein in isolated human lymphocytes. *J. Exp. Med.* 145:1531.
12. Iscove, N. N., and F. Melchers. 1978. Complete replacement of serum by albumin, transferrin, and soybean lipid in cultures of lipopolysaccharide-reactive B lymphocytes. *J. Exp. Med.* 147:923.
13. Brown, M. S., and J. L. Goldstein. 1986. A receptor-mediated pathway for cholesterol homeostasis. *Science (Wash. DC)* 232:34.
14. Barnes, D., and G. H. Sato. 1980. Methods for growth of cultured cell in serum-free medium. *Anal. Biochem.* 102:255.
15. Barnes, D., and G. H. Sato. 1980. Serum-free cell culture: a unifying approach. *Cell.* 22:649.
16. Sato, G. H., A. B. Pardee, and D. A. Sirbasku. 1982. Cold Spring Harbor Conferences on Cell Proliferation. Vol. 9. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 1214 pp.
17. Esfahani, M., L. Scerbo, and T. M. Devlin. 1984. A requirement for cholesterol and its structural features for a human macrophage-like cell line. *J. Cell. Biochem.* 25:87.
18. Chen, J.-K., T. Okamoto, J. D. Sato, G. H. Sato, and D. B. McClure. 1986. Biochemical characterization of the cholesterol-dependent growth of the NS-1 mouse myeloma cell line. *Exp. Cell Res.* 163:117.
19. Kinsky, S. C. 1970. Antibiotic interaction with model membranes. *Annu. Rev. Pharmacol.* 10:119.
20. Saito, Y., S. M. Chou, and D. F. Silbert. 1977. Animal cell mutants defective in sterol metabolism: a specific selection procedure and partial characterization of defects. *Proc. Natl. Acad. Sci. USA.* 74:3730.
21. Chang, T.-Y., and C. C. Y. Chang. 1982. Revertants of a Chinese hamster ovary cell mutant resistant to suppression by an analogue of cholesterol: isolation and partial biochemical characterization. *Biochemistry.* 21:5316.