PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 149, 439–442 (1975)

Role of Bovine Albumin in a Serum-free Suspension Cell Culture Medium (38823)

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Bovine serum albumin (BSA) can be substituted in place of whole serum to provide the necessary growth-enhancing factors for the *in vitro* propagation of mammalian cells (1–6). Other investigators have reported the role of albumin to be protective as a result of its "capacity and ability to trap toxic substances in the culture medium" (7). Recently, we have formulated a serum-free suspension culture medium for tumor cells. The medium contains commercial bovine serum albumin V in which bound fatty acids are suspected to play an important role in cell growth. This report deals with the growth of different mammalian tumor cells in serum-free medium and our considerations pertaining to the possible role of BSA as a serum substitute.

Materials and Methods. Basal medium and BSA. In addition to BSA, Eagle's MEM was supplemented with sodium pyruvate, insulin, putrescine, zinc sulfate, and various nucleosides (Yamane *et al.*, in press). All components were dissolved in deionized distilled water and sterilized through membrane filters. Commercial preparations of BSA include lot numbers E29506(E), F22802(F), G35908(G) (Armour Pharmaceutical Company, Chicago, IL), and lot numbers 66 and 14 (the latter are designated as being "fatty acid free," Pentex Inc., Kankakee, IL). All BSA stock preparations were dissolved in the basal medium at a final concentration of 10%, neutralized with 1 *N* NaOH, sterilized by membrane filtration, and then added to the sterile basal medium in appropriate amounts.

Growth experiments. Yoshida ascites sarcoma cells and ascites hepatoma cells were isolated from rat peritoneal fluid by means of a syringe puncture (8). The cells were pelleted, diluted with basal medium, and placed *in vitro*. Cells from established cell lines and secondary cultures, initially isolated with Eagle's MEM supplemented with 10% calf serum, were diluted and transferred to the serum-free medium. All cell cultures were maintained in plastic Petri dishes (diameter 3.5 cm Falcon Plastic Co. CA) with a final test medium volume of 1.5 ml., and held at 37°C in a CO_2-incubator for 3–5 days without subsequent medium changes. Control cultures were maintained in Eagle's MEM supplemented with 10% calf serum (11). Cell proliferation was determined by hemocytometer counts at the end of the incubation period(s).

Fatty acid analysis of BSA preparations. The lipid in BSA preparations was extracted with chloroform–methanol according to the procedure outlined by Morrison *et al.* (9). The extracts were pooled, washed with water to remove nonlipid material, and concentrated by evaporation of the solvent under nitrogen gas. Total extracted lipids were methylated and fatty acid methyl esters were measured by gas chromatography and compared with the internal standard, malgaric acid (9).

Results and Discussion. Growth of Yoshida sarcoma cells in serum-free medium supplemented with BSA. The various preparations of BSA were added to the basal medium at concentrations ranging from 0.1 to 3.0%. The 1% concentration of BSA proved to be optimal for the growth of primary explants of Yoshida sarcoma cells, even though the growth-promoting activity was different for each BSA preparation. Recently, it was found that the addition of 0.1% BSA, 0.1% Methocel 15 CPS (Dow Chemicals, Co., MI), and certain fatty acids to basal medium supported the growth of Yoshida cells to the same degree as media supplemented only with 1.0% BSA. The cells grew equally well in both the serum-free and serum-containing media, even at concentrations as low as 3×10^2 cells per ml. Also, microscopically con-

firmed single cell transfers to individual wells of microtiter plates proliferated uniformly. Cells cultured in serum-free medium were transferred serially for 90 passages during the course of these studies which covered a period of more than 1 yr. Karyological monitoring of the Yoshida sarcoma cells failed to disclose any changes in the modal number of 40 chromosomes: the number observed *in vivo*. Yet, the modal number of cells cultured in serum-containing medium had increased to 41.

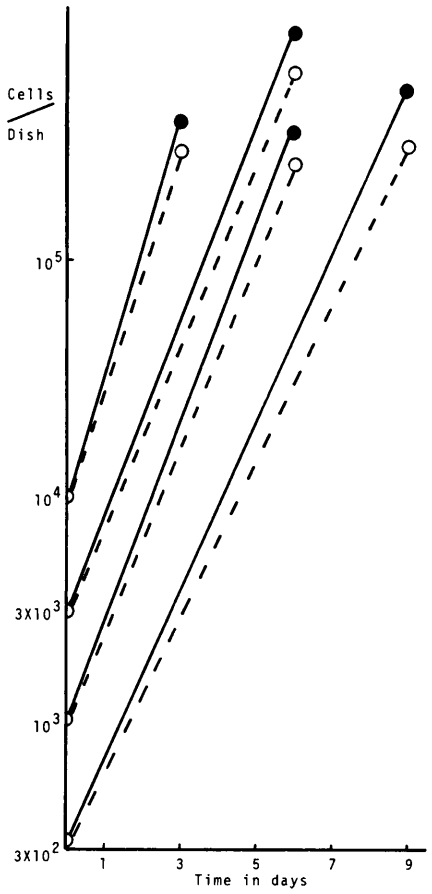

FIG. 1. Growth curves of Yoshida sarcoma primary cultures in both serum-free and serum-containing media. Dotted lines: cell growth in MEM supplemented with 10% calf serum; straight lines: cell growth in BSA-supplemented serum-free medium (see text for details).

The growth and characters of other primary and continuous cultures. Table I lists seven other types of tumor cells, isolated from animal hosts, which grew equally well in both the serum-free and serum-containing media while passaged serially over a period of more than 1 yr. Also, six additional established cell lines and two secondary tumor cell cultures grew in the serum-free medium. Likewise, human peripheral blood lymphocytes underwent transformation in the presence of phytohemagglutinin, as demonstrated by the incorporation of ^{3}H-thymidine. Growth curves of four representative cell cultures are presented in Fig. 2. One of the characteristic phenotypes of the AH66F ascites hepatoma cells is the presence of glycogen granules in the cytoplasm. This prominent feature was retained in the course of five consecutive transfers. The sparseness or absence of glycogen in AH310 cells persisted for a similar period of time and transfers. The above findings indicate that the BSA-supplemented, serum-free culture medium can be employed routinely for suspension-type cultures of ascites tumor cells and lymphocyte transformation.

The role of BSA in cell growth. Figure 3 illustrates the growth of Yoshida sarcoma cells in two different BSA-containing culture media (one was supplemented with BSA [Armour G], while the other was formulated

TABLE I. TYPES OF CELLS GROWN IN SERUM-FREE MEDIUM.

Animal species	Tumor cells	Culture conditions
Rat	Yoshida sarcoma, ascites hepatoma AH13, AH66, AH130, AH7974	Primary and transfer
	Morris hepatoma 7288C	Established
Mouse	Mammary ascites tumor FM3A	Primary and transfer
	Ehrlich tumor, MOPC31B myeloma	Secondary and transfer
	Leukemia L5178Y	Established
Human	Burkitt lymphoma P3HR-1, Daudi lymphocytes NC37	Established
	Peripheral blood lymphocyte	Primary

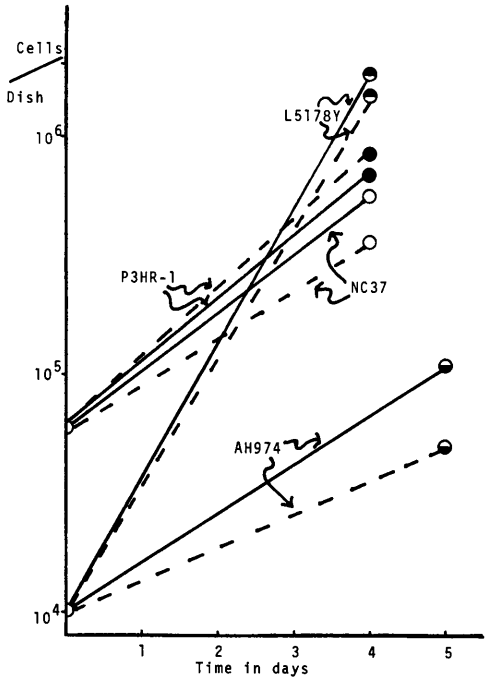

FIG. 2. Growth curves of L5178Y, P3HR-1, NC37, and AH7974 cell lines in the serum-free and serum-containing media. Straight lines: growth curves in serum-free BSA medium; dotted lines: growth curves in serum-containing medium.

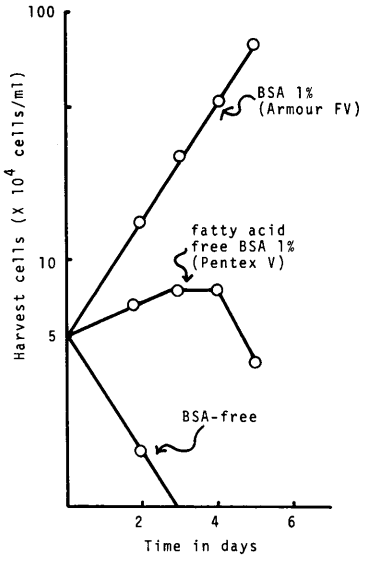

FIG. 3. Growth curves of Yoshida sarcoma primary cells in different serum-free BSA media (one supplemented with commercial BSA, the other prepared with commercial fatty acid-free BSA), and in serum- and BSA-free medium.

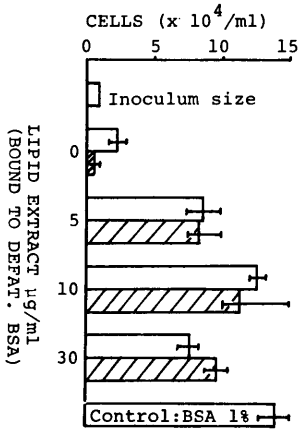

FIG. 4. The effect of reconstituted BSA (lipid extract plus 1% defatted BSA) on cell growth. Each column indicates the growth of Yoshida sarcoma cells in media containing various amounts of the lipid extract after 3 days of incubation. Shaded bars: cell harvests with commercial fatty acid-free BSA (Pentex V); unshaded bars: cell harvests with defatted BSA.

with commercial fatty acid-free BSA, described as having been defatted by the use of active charcoal following Chen's methods [10]), and BSA-free medium. As shown in the Figure, the BSA-free and the fatty acid-free BSA-supplemented media, failed to support growth. Similar results were obtained for BSA defatted with methanol–chloroform. It was also noted that the most effective growth-promoting lot of BSA, Armour G, had the highest content of oleic and linoleic acids among the lots examined. These determinations, based on gas-chromatographic analyses of methanol–chloroform extracts, provided a basis for designating, by means of reconstitution experiments, which component(s) in the different lots of BSA provided the growth-promoting potential. The lipid material extracted from BSA (Armour G) was supplemented separately with commercial fatty acid-free BSA and BSA defatted with organic solvent, as described by Morrison *et al.* (9). The defatted BSA-supplemented lipid extract was added to basal medium at a concentration of 1%, and the growth-enhancing potential of this reconstituted BSA was examined with Yoshida sarcoma cells (Fig. 4).

Cell growth with reconstituted BSA was somewhat inferior to that noted for the un-

extracted BSA. In fact, the lipid extract was quite inhibitory in the absence of defatted BSA. Furthermore, when sodium salts of oleic and linoleic acid (10 μg each) were added to 10 mg of defatted BSA, before supplementing the basal medium in the amount of 1% BSA, cell growth approximated 70% of that noted in control cultures. These results strongly indicate that the albumin-bound lipid content in commercial lots of BSA plays an important role in cell growth when employing serum-free medium, even though Ham (12) indicated that certain free fatty acids can replace BSA for the clonal growth of Chinese hamster cells.

Summary. A serum-free culture medium, supplemented with 1% bovine serum albumin, supported the growth of both primary and continuous suspension-type cultures of various mammalian tumor cells. The role of albumin added to the medium was also studied. Defatted albumin failed to support cell growth, unless reconstituted with its lipid extract. Similarly, defatted albumin, when combined with oleic and linoleic acids, also supported cell growth. Therefore, albumin-bound fatty acids play an important growth-promoting role in serum-free medium.

We thank the Nissui Saiyaku Company for supplying the fatty acids. This work was partly supported by a Grant-in-Aid from the Educational Ministry of Japan.

1. Fisher, H. W., Puck, T. T., and Sato, G. J., Exp. Med. **109,** 649 (1959).
2. Michl, J., Exp. Cell Res. **23,** 324 (1961).
3. Todaro, G. J., and Green, H., Proc. Soc. Exp. Biol. Med. **116,** 688 (1964).
4. Moskowitz, M., and Schenck, D. M., Exp. Cell Res. **38,** 523 (1965).
5. Ham, R. G., Proc. Nat. Acad. Sci. USA **53,** 275 (1965).
6. Matsuya, Y., and Yamane, I., Proc. Soc. Exp. Biol. Med. **135,** 893 (1970).
7. Temin, H. M., Pierson, R. W., and Dulak, N. C., *in* "Growth, Nutrition and Metabolism of Cells in Culture" (G. H. Rothblat and V. J. Cristofalo, eds.), Vol. 1, p. 55. Academic Press, New York (1972).
8. Yoshida, T., Muta, Y., and Sasaki, Z., Proc. Imp. Acad. Tokyo **20,** 611 (1944).
9. Morrison, S. J., and Jenkin, H. M., In Vitro **8,** 94 (1972).
10. Chen, R. F., J. Biol. Chem. **242,** 173 (1967).
11. Eagle, H., Science **130,** 432 (1959).
12. Ham, R. G., Science **140,** 820, (1963).

Received Sept. 17, 1974. P.S.E.B.M., 1975, Vol. 149