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Role of Bovine Serum Albumin in Blastoid Transformation of Lymphocytes by Phytohemagglutinin (39690)

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Human peripheral lymphocytes are activated to undergo blast transformation by specific and nonspecific stimulants *in vitro* (1-3). Supplementation with autologous serum or fetal calf serum to the medium is generally required for the transformation of lymphocytes. However, the factor(s) in serum which cause blast transformation of the stimulated lymphocytes are still unknown. The study of such factors in serum may reveal the mechanism of activation and proliferation of the stimulated lymphocytes. Polet and Spieker-Polet and others demonstrated that serum albumin itself was indispensable to the medium component for the blast transformation of lymphocytes by mitogens (4-6).

Recently, Yamane *et al.* have formulated a serum-free culture medium (SF medium) in which various mammalian tumor cells including a human lymphoid cell type can grow well (7). With this SF medium, albumin-bound fatty acids were found to play an important role in cell culture. Therefore, we undertook to investigate mitogen-induced transformation of human lymphocytes in this medium to see whether the albumin-bound fatty acids play a significant role in the transformation of lymphocytes. As a result, an important role of fatty acids in the phytohemagglutinin (PHA)-stimulated transformation of the lymphocytes was established.

Materials and methods. Basal medium and BSA. As described previously by Yamane (7) for the preparation of basal SF medium, Eagle's minimum essential medium (MEM) was supplemented with sodium pyruvate, insulin, putrescine, zinc sulfate, and various nucleosides. All components were dissolved in deionized distilled water and then sterilized through Millipore membrane filters. BSA (Cohn Fr.V) and defatted preparations of BSA were purchased from Armour Pharmaceutical Co. (Chicago, Ill.), Pentex Inc. (Kankakee, Ill.), and Sigma Chemical Co. (St. Louis, Mo.). These BSAs were dissolved in the basal medium, neutralized with 1 *N* NaOH, and then sterilized through the membrane filters. Oleic and linoleic acids were first dissolved in a small amount of ethanol and then diluted to a suitable concentration with culture medium.

Preparation of lymphocytes. Heparinized peripheral blood was obtained from healthy adults. The blood was mixed with one-third volume of 3% gelatin (Difco, Detroit, Mich.) in phosphate-buffered saline (PBS) as described by Coulson and Chalmers (8). The mixture was kept for 45 min at 37° to sediment the erythrocytes. The leukocyte-enriched plasma was collected and washed twice with Hanks' balanced salt solution containing 0.1% gelatin and then suspended in the culture medium. The viable cells were counted by trypan blue dye exclusion tests and adjusted to 5×10^5 cells/ml. These cell preparations usually contained 60-70% lymphocytes as detected by Giemsa staining and had more than 96% viability.

Cell culture. Lymphocytes were cultured in 6 × 100-mm round-bottomed glass tubes with loosely fitted aluminum caps. Each tube contained 1 ml of tissue culture medium with 5×10^5 lymphocytes. These suspensions were cultured at 37° in an atmosphere of 5% CO_2 and 95% air. Phytohemagglutinin (PHA-P, Difco, Detroit, Mich.) was reconstituted with 5 ml of distilled water and diluted with the medium.

Assay of DNA synthesis. One microcurie of tritiated thymidine (New England Nuclear Inc., Boston, Mass.; specific activity, 10 ci/mmol) was added to the culture 4 hr prior to termination of incubation. After pulsing, these samples were chilled and washed with cold PBS, and then one drop of 1% bovine serum albumin and 5 ml of 10%

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trichloroacetic acid (TCA) were added to each tube. After a 2-hr incubation at 4°, the tubes were centrifuged, and resulting precipitates were washed twice with 5% TCA. The precipitates were dissolved in ethanol and then centrifuged. The pellets were hydrolyzed with 0.2 ml of 2 *N* sodium hydroxide at 80° for 2 min, and one drop of 4% $SnCl_2 \cdot 2H_2O$ was added. The samples were dissolved in 0.6 ml of Biosolv II (Beckman) and 10 ml of scintillation fluid (2,5-diphenyloxazole, PPO, and 1,4-bis[2-(5-phenyloxazolyl)]-benzene, POPOP) was added. The solution was counted in a Packard scintillation counter. The resulting counts were corrected to values of disintegration per minute (dpm) by referring to a quench curve established for the machine. Standard deviations were calculated from the values of triplicate tubes. The stimulation index was calculated with the following formula:

$$\text{Stimulation index (SI)} = \frac{\text{dpm of stimulated cells}}{\text{dpm of unstimulated cells.}}$$

Results. Optimal concentrations of BSA in SF medium. To determine the optimal concentrations of supplementary BSA necessary for SF medium in lymphocyte transformation by PHA, the basal SF medium was supplemented with four different concentrations ranging from 1.0 to 0.125% BSA and used for the lymphocyte transformation. As shown in Table I, addition of 1.0 to 0.25% BSA to the medium was found to be highly effective in inducing the PHA response of the lymphocytes ($P < 0.001$), whereas the addition of 0.125% BSA gave the response only at a level similar to that in BSA-free medium. On the other hand, in the absence of PHA, [^{3}H]thymidine incorporation in all the media containing different concentrations of BSA with the exception of 1.0% BSA was much lower than was the case with BSA-free medium. These results clearly show that extremely high stimulation indices could be obtained with the culture containing 0.25 and 0.5% BSA. Thus, for all subsequent experiments, SF medium supplemented with 0.25% BSA was used.

Time course of the lymphocyte transformation in SF medium. Lymphocyte transformations by different concentrations of PHA in SF medium and RPMI 1640 medium supplemented with 10% fetal calf serum during a period of 120 hr are summarized in Fig. 1. In RPMI 1640 medium, DNA synthesis began 24–48 hr after incubation and reached a maximum at 72 hr, whereas synthesis in SF medium began 48–72 hr and reached its maximum after 96 hr of incubation. The maximum value of DNA synthesis in SF medium was about four times higher than that in RPMI 1640 medium. The results also show that a high level of DNA synthesis was observed even with the lymphocytes in SF medium stimulated by PHA at a concentration as low as 0.6 μg/ml.

No effects of defatted BSA on lymphocyte transformation. It has been shown by one of the authors and his colleagues (7) that the BSA-bound fatty acids may play an important role in the growth of tumor cells in SF medium. Therefore, in order to determine whether the fatty acids have the same function on lymphocyte proliferation, we tested the PHA-stimulated transformation of lymphocytes in SF medium supplemented with

TABLE I. EFFECTS OF BSA CONCENTRATION ON DNA SYNTHESIS OF PHA-STIMULATED LYMPHOCYTES IN SERUM-FREE MEDIUM.[a]

Concentration of BSA (%)	[^{3}H]thymidine incorporation		Stimulation index
	PHA	Control	
1.0	359,224 ± 6,591[b]	3,092 ± 390	119.7
0.5	433,050 ± 17,871	746 ± 104	580.5
0.25	378,910 ± 11,741	383 ± 42	989.3
0.125	18,876 ± 2,876	389 ± 35	48.5
—	14,945 ± 2,690	1,989 ± 258	7.5

[a] In the basal SF media 5×10^5 lymphocytes supplemented with various concentrations of BSA (Armour) were incubated with 3.0 μg/ml of PHA or without PHA.

[b] Values represent the mean disintegrations per minute ± SD of incorporated [^{3}H]thymidine in triplicate cultures.

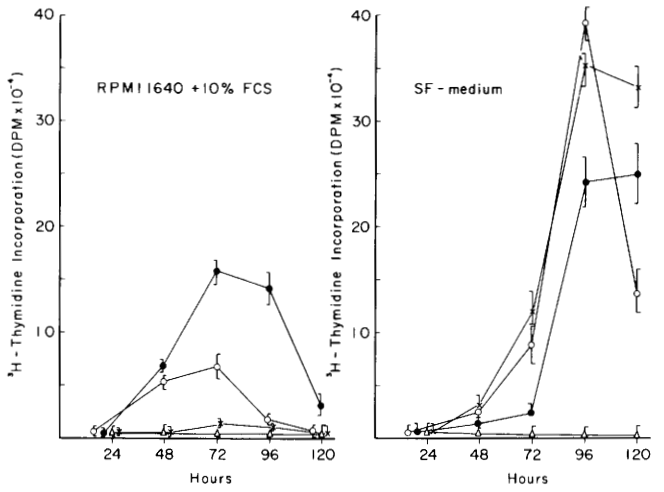

FIG. 1. Kinetics of PHA stimulation of human peripheral lymphocytes (5×10^5 cells) in SF medium and RPMI 1640 with 10% fetal calf serum. ●——●, 15.0 μg/ml of PHA; ○——○, 3.0 μg/ml of PHA; x——x, 0.6 μg/ml of PHA; △——△, no mitogen.

defatted BSA and compared the results with those in SF medium containing the native BSA. Different commercial preparations of native or defatted BSA (0.25%) were added to the basal SF medium and examined for their effect on the PHA response of the lymphocytes at a 96-hr incubation (Table II). In addition, transformation of lymphocytes by various concentrations of PHA in SF medium supplemented with 0.25% Armour's native or defatted BSA were measured at different incubation times (Fig. 2). Both the experiments clearly show that only one-tenth or less response to PHA of the lymphocytes was obtained in the SF medium supplemented with any preparations of defatted BSA. The decrease of medium pH was observed only with the culture in which a high level of incorporation of [^{3}H]thymidine was demonstrated.

We next examined the effect of oleic and linoleic acids on the PHA-stimulated transformation of lymphocytes. Both the fatty acids at concentrations of 1–4 μg/ml were added to the medium containing defatted BSA and tested for their effect on the PHA response of the lymphocytes. As shown in Fig. 3, addition of 1 and 2 μg/ml of fatty acids restored the transformation of lymphocytes to 60 and 75% of the control value, respectively, although high concentrations of the drugs (3 and 4 μg/ml) had no restoring effect on lymphocyte transformation and showed a somewhat inhibitory ef-

TABLE II. EFFECTS OF BSA AND DEFFATED BSA ON DNA SYNTHESIS OF PHA-STIMULATED LYMPHOCYTES.[a]

BSA	[^{3}H]thymidine incorporation		Stimulation index
	PHA	Control	
BSA (Fr.V)			
Armour	267,025 ± 32,043[b]	387 ± 56	690
Pentex	290,822 ± 18,868	375 ± 42	775
Sigma	328,345 ± 14,857	605 ± 37	543
Defatted BSA			
Armour	9,425 ± 1,074	161 ± 28	59
Pentex	11,094 ± 1,818	1,051 ± 156	11
Sigma	9,857 ± 1,003	409 ± 29	24

[a] In the basal SF media 5×10^5 of lymphocytes with 0.25% different commercial native or defatted BSAs were incubated with 3.0 μg/ml of PHA or without PHA.

[b] Values represent the mean disintegrations per minute ± SD of incorporated [^{3}H]thymidine in four cultures.

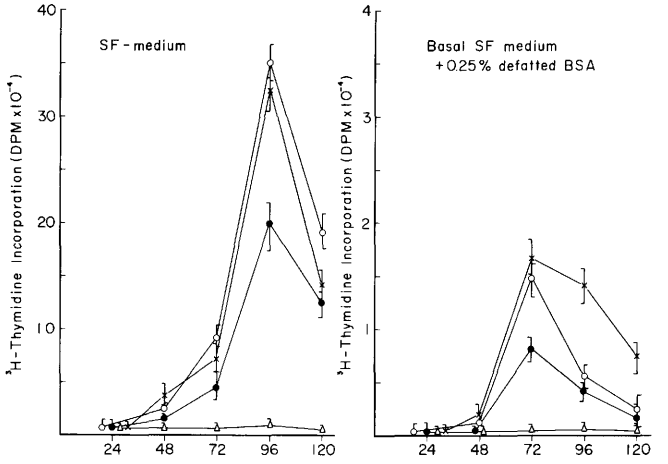

FIG. 2. Kinetics of PHA stimulation of human peripheral lymphocytes (5×10^5 cells) in the basal SF medium with 0.25% BSA and 0.25% defatted BSA. ●——●, 15.0 μg/ml of PHA; ○——○, 3.0 μg/ml of PHA; x——x, 0.6 μg/ml of PHA; △——△, no mitogen.

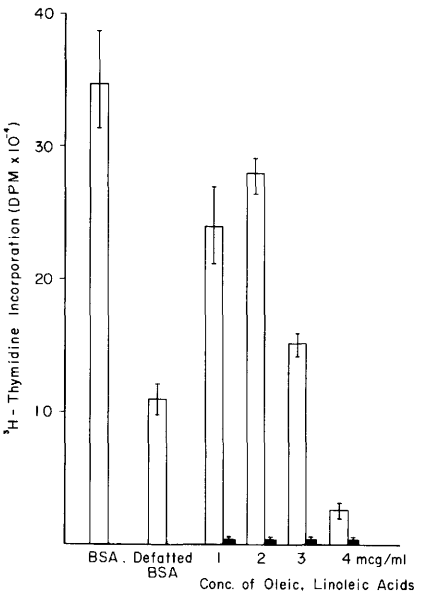

FIG. 3. The effects of oleic and linoleic acids on the DNA synthesis of PHA-stimulated lymphocytes. Lymphocytes, 5×10^5, were incubated with 3.0 μg/ml of PHA for 96 hr. □, [^{3}H]thymidine incorporation in the medium supplemented with 0.25% BSA or defatted BSA with oleic and linoleic acids; ■, [^{3}H]thymidine incorporation in the basal SF medium with oleic and linoleic acids.

fect. However, these fatty acids when added to the BSA-free basal medium showed no effect on the transformation (Fig. 3). These results clearly indicate that fatty acids may be indispensable to the medium component for the blast transformation of PHA-stimulated lymphocytes, although the collaboration of albumin with fatty acids may be needed for the transformation.

Discussion. SF medium formulated by Yamane *et al.* (7) has been shown to support the growth of tumor cells as well as or more abundantly than conventional media containing the serum. In the present experiments, it is clearly shown that this SF medium is also useful for the assay of DNA synthesis by PHA-stimulated lymphocytes. The results demonstrate the following characteristics of SF medium as a culture medium for lymphocyte transformation experiments: (a) remarkable response to lower PHA doses, (b) high incorporation of [^{3}H]thymidine into the stimulated lymphocytes, and (c) low background resulting in a higher stimulation index.

One of the present authors has shown earlier that defatted albumin, unless reconstituted with its lipid extracts or oleic and linoleic acids, failed to support tumor cell growth in SF medium. Our experiments also clearly demonstrate that the albumin-bound fatty acids play an important role in transformation of the lymphocytes. By contrast, Polet and Spieker-Polet have reported that fatty acids may not be necessary for the transformation of lymphocytes stimulated by concanavalin A (Con A) (4, 5). The exact explanation for this discrepancy in the role of fatty acids in the transformation of lymphocytes is not clear. However, Polet

and Spieker-Polet performed the experiments in which Con A-treated lymphocytes were washed once with the medium containing 10% fetal calf serum, washed only once with the protein-free medium to remove free Con A, and then added to the fatty acid-free medium for the estimation of DNA synthesis. This procedure might allow binding to the cell surface of fatty acids derived from the fetal calf serum. Washing such cells only once also would not be sufficient to remove the cell-bound fatty acids. Therefore, it should be considered that the cells which retained fatty acids supported the transformation of lymphocytes, even if the fatty acids were not supplemented with the estimation medium.

The exact mechanisms of this augmentation by fatty acids of PHA-stimulated DNA synthesis of lymphocytes are not clear. However, it is well known that fatty acids in general play an important part in the physiological stability of cellular membranes of mammalian cells such as Ehrlich cells, polymorphonuclear leukocytes, and alveolar macrophages (9). Therefore, we presume that the fatty acids in this case also may play a part in the stabilization of lymphocyte and/or macrophage membranes.

Overall, it appears that the SF medium used in this work may be useful as a culture medium not only to test the mitogenic response of human lymphocytes for clinical studies but also to investigate the role of fatty acids in lymphocyte differentiation.

Summary. Employing a serum-free medium (SF medium), blast transformation of human peripheral lymphocytes stimulated by PHA was investigated. A remarkable PHA response with extremely high stimulation indices was obtained in SF medium containing 0.25–0.5% BSA. However, lymphocytes were unresponsive to PHA in the medium containing defatted BSA, unless supplied with oleic and linoleic acids. It is concluded that the albumin-bound fatty acids play an important role in lymphocyte transformation in SF medium, as observed in the case of tumor cell cultivation.

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