Demonstration of the Requirements for Cholesterol or Low Density Lipoprotein in the Antibody Response of Murine Lymphocytes in Serum-free Culture

Hitoshi OHMORI[†] and Shigehiro TAKAHAMA*

Department of Biotechnology, Faculty of Engineering, Okayama University,
Tsushima-Naka, Okayama 700, Japan

*Teijin Institute for Biomedical Research, Hino,
Tokyo 191, Japan

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We report the essential role of lipid in the development of a serum-free culture medium that supports the primary antibody response by murine lymphocytes *in vitro*. In our preceding paper, we have reported that β -cyclodextrin (β -CD) is effective as a serum-substitute in murine lymphocyte cultures [H. Ohmori and I. Yamamoto, *Eur. J. Immunol.*, 17, 79 (1987)]. At first, we employed RPMI-1640 that was supplemented with fatty acid-free albumin, insulin, transferrin, and β -CD as the basal serum-free medium (BSF medium). BSF medium supported the antibody response less than 30 % as efficiently as 10 % fetal calf serum (FCS)-containing medium. When ether-extracts of FCS (FCS ex.) was added to BSF medium, the antibody response was enhanced by approximately 2-fold. FCS ex. was purified by silica gel thin-layer chromatography. The analyses of the purified active component by high performance liquid chromatography and mass spectrometry revealed it to be cholesterol. Authentic cholesterol or low density lipoprotein enhanced the antibody response as efficiently as FCS ex. in BSF medium. The same level of the antibody response as that in 10 % FCS-containing medium was attained when low density lipoprotein was added to BSF medium in combination with L-alanine, putrescine, and pyruvate.

Culture conditions has been reported for differentiating murine spleen lymphocytes into antibody-forming cells when the cells were cultured with an antigen *in vitro*.^{1,2)} The *in vitro* antibody response is a useful experimental system for analyzing the molecular and cellular mechanisms of immune responses. Lymphocyte cultures for *in vitro* antibody responses are usually carried out in RPMI-1640 medium containing 10% fetal calf serum (FCS).^{3,4)} However, one must select a good batch of FCS for inducing a high level of the antibody response. In addition, it has often been pointed out that contradictory obser-

vations were made in the same type of experiments depending on which batch of FCS was used in the lymphocyte culture.^{5,6)} For avoiding these problems, the use of a serum-free culture medium has been recommended. Although serum-free media for inducing *in vitro* antibody responses have been reported by several investigators,⁷⁻⁹⁾ these media have been reported to be inadequate for inducing primary antibody responses because the responses in these media were often unstable due to undefined factors.⁷⁾

We have recently reported that β -cyclodextrin (β -CD) is effective as a serum-substitute

Abbreviations: BSA, bovine serum albumin; BSF medium, basal serum-free RPMI-1640 medium; β -CD, β -cyclodextrin; FCS, fetal calf serum; FCS ex., ether-extracts of FCS; LDL, low density lipoprotein; MEM, minimum essential medium; PFC, plaque-forming cells; SRBC, sheep erythrocytes.

[†] To whom all correspondence should be addressed.

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for inducing the primary antibody response to sheep erythrocytes (SRBC) in murine lymphocyte cultures.^{10,11} Interestingly, FCS content could be reduced from 10% to 1% without abrogating the antibody response if β -CD was present in the medium. Therefore, a serum-free culture medium for the lymphocyte culture can be developed provided that this 1% FCS can be replaced by some defined components. In this report, we isolated a factor from the ether-extracts of FCS (FCS ex.) that enhanced the antibody response under serum-free culture conditions, and identified it as cholesterol. We also described that cholesterol or low density lipoprotein (LDL) was shown to be essential for developing the serum-free medium for murine lymphocyte cultures.

Materials and Methods

Materials. Female BALB/c mice (8 weeks of age) were purchased from Shizuoka Laboratory Animal Center (Hamamatsu, Japan), and they were used at 10~15 weeks of age.

Chemicals and other materials were obtained from the following commercial sources; 2-mercaptoethanol, β -CD, fatty acid-free bovine serum albumin (BSA), bovine insulin, human transferrin, human LDL and cholesterol (Sigma Chemical Co., St. Louis, MO); L-alanine and putrescine dihydrochloride (Nacalai tesque, Kyoto, Japan); 100 mM sodium pyruvate solution and FCS (Flow Laboratories, McLean, VA); SRBC (Nishinippon Sheep Farm, Fukuyama, Japan); RPMI-1640 and Eagle's minimum essential medium (MEM) (Nissui Seiyaku, Tokyo, Japan); guinea pig complement (Kyokuto Seiyaku, Tokyo, Japan).

Culture media. RPMI-1640 medium was supplemented with 10% FCS and 1×10^{-5} M 2-mercaptoethanol. Selected batches of FCS that supported the antibody response efficiently were used. Basal serum-free RPMI-1640 medium (BSF medium) contained 250 μ g/ml β -CD, 500 μ g/ml fatty acid-free BSA, 5 μ g/ml insulin, 50 μ g/ml transferrin, and 1×10^{-5} M 2-mercaptoethanol as serum-substitutes. These media were usually supplemented with 1.5 mg/ml sodium bicarbonate, 100 U/ml penicillin G, and 100 μ g/ml streptomycin.

Antibody response in vitro. Spleens of BALB/c mice were used as the source of murine lymphocytes. Dissociated spleen cells (6.5×10^6) were cultured in duplicate or triplicate with 2×10^6 SRBC in 1 ml of serum-free or 10% FCS-containing RPMI-1640 medium. Cultures were

maintained at 37°C for 5 days in a humidified atmosphere of 5% CO₂ and 95% air using 24-well trays (NUNC, Roskilde, Denmark). Anti-SRBC antibody-forming cells were assayed by enumerating plaque-forming cells (PFC) according to the hemolytic plaque assay of Jerne and Nordin¹² with some modifications. Briefly, cultured cells were harvested, washed once with 3 ml MEM, and suspended in 2 ml MEM. A hundred μ l of the cell suspension and 20 μ l of 10 fold-diluted guinea pig complement were mixed with 2 ml MEM containing 0.8% agarose and 1% SRBC that had been maintained at 45°C. The mixture were immediately spread over plastic petri dishes (diameter, 90 mm), and incubated at 37°C for 3 hr. The number of PFC were measured by counting developed hemolytic plaques. Typical results from several repeated experiments are presented.

Preparation of FCS ex. One ml of FCS was mixed vigorously with the same volume of a mixture of ether and ethanol (4:1). After centrifugation at 2000 rpm for 5 min, the organic layer was collected, and evaporated *in vacuo* to dryness. The residue was first dissolved in 50 μ l of RPMI-1640 medium containing 500 μ g/ml fatty acid-free BSA. Before being added to the culture medium, these preparations were sterilized by passing through a 0.22- μ m membrane filter. Usually 10 μ l of this FCS ex. preparation was added to 1 ml of culture medium. Cholesterol and every organic solvent-soluble fraction obtained during purification of FCS ex. were dissolved in the same manner.

Results and Discussion

FCS ex. contains a factor that stimulates the antibody response to SRBC under serum-free culture conditions

For inducing the primary antibody response to SRBC *in vitro* in murine lymphocytes, the cells are usually cultured in 10% FCS-containing RPMI-1640 medium. As reported previously,¹⁰ the antibody response was abrogated when FCS was reduced from 10% to 1%, but restored to the original level when β -CD was added as shown in Fig. 1 (Exp.1). Then, we attempted to replace this 1% FCS with some defined factors in order to establish serum-free culture conditions. As a first stage, we devised BSF medium that consists of RPMI-1640, fatty acid-free BSA, insulin, transferrin, and β -CD (see **Materials and Methods**). BSF medium supported the antibody response less than 30% as efficiently as 10% FCS-containing medium as illustrated in Fig. 1 (Exp. 2). When FCS ex. was added to

Fig. 1. Enhancing Effects of FCS ex. on the Antibody Response Elicited in BSF Medium.

BALB/c spleen cells (6.5×10^6) were cultured with 2×10^6 SRBC in 1 ml of RPMI-1640 containing 1 or 10% FCS or in BSF medium. Where indicated, β -CD (250 $\mu\text{g/ml}$) was added to 1% FCS-containing medium. After 5 days, generated PFC were enumerated by the method of Jerne and Nordin¹²⁾ (see **Materials and Methods**). BSF medium is RPMI-1640 supplemented with fatty acid-free BSA, insulin, transferrin, and β -CD at concentrations described under **Materials and Methods**. FCS ex. means ether-extracts of FCS. The data were presented as the mean number of PFC $\pm$ standard error from triplicate experiments.

BSF medium at the concentration expected to be equivalent to that contained in 1% FCS, the response was found to be augmented significantly. The responses in RPMI-1640 medium containing 1% FCS + β -CD were approximately the same as those in BSF medium containing 1% FCS. Thus, the antibody response under the former culture conditions were sometimes used as a control in this and the following experiments.

Under these culture conditions, the antibody response was strictly dependent on β -CD in the BSF medium as shown in Fig. 2. Although the mode of action of β -CD remains to be elucidated, it did not stimulate antibody responses nonspecifically.¹⁰⁾ The ability to form inclusion complexes appears to be involved in supporting the antibody response because maltoheptaose, which is a linear analogue of β -CD and is not able to form inclusion complexes, was inactive.¹⁰⁾ It has been pointed out that one of the vital roles of FCS in the cell culture is to adsorb toxic substances that were formed during cultures onto its protein com-

Fig. 2. Dependence of the Antibody Response on β -CD in the Serum-free Medium.

β -CD was added at the indicated concentrations to serum-free RPMI-1640 containing fatty acid-free BSA, insulin, transferrin, and FCS ex. The data were presented as the mean number of PFC $\pm$ standard error from triplicate experiments.

ponents.¹³⁾ β -CD might be able to substitute for this role of FCS. Yamane *et al.*¹⁴⁾ have demonstrated that α -CD complexed with oleic acid can replace fatty acid-containing BSA as a lipid source in the serum-free culture of human diploid fibroblasts. However, α -CD or γ -CD were less than 20% as active as β -CD in our experiments.¹⁰⁾ The role of β -CD in our case would be different from that in the culture system of Yamane *et al.* because the β -CD requirement was still observed even in the presence of BSA (Fig. 2).

Identification of the active substance in FCS ex. as cholesterol

FCS ex. was developed on silica gel thin-layer plates as illustrated in Fig. 3. Each spot was extracted and examined for its enhancing effects in the antibody response by adding to BSF medium. Figure 3 clearly demonstrated that spot C was the most active. Since the *R_f* value of spot C resembled that of cholesterol,¹⁵⁾ spot C component was extracted, and analyzed by high performance liquid chromatography (HPLC) as illustrated in Fig. 4, showing that the substance was eluted as a single peak with the same retention time as that of authentic cholesterol. The peak component was collected, and analyzed by mass spectrometry as shown in Fig. 5. It was found that every peak including 386 (M^+) coincided with those

Fig. 3. Fractionation of FCS ex. by Silica Gel Thin-Layer Chromatography.

FCS ex. was developed in duplicate on silica gel thin-layer plates with CHCl_3 -MeOH- H_2O (100:35:3). Organic compounds were located on one plate by spraying concentrated H_2SO_4 , then heating. The area corresponding to each spot was scratched off of another plate, and extracted with CHCl_3 -MeOH (2:1). The activities of each fraction was assayed by adding to BSF medium as described in **Materials and Methods**. The data were presented as the mean number of PFC from duplicate experiments. The experimental variations between duplicate cultures did not exceed 15% of the mean values.

Fig. 4. Analysis of Spot C Component by HPLC.

Authentic cholesterol and/or spot C component (see Fig. 3) was analyzed by HPLC using a Lichrosorb Si60 column. Elution was done with *n*-hexane-isopropyl alcohol (98:2), and the elution profiles were recorded at 210 nm. I, authentic cholesterol; II, spot C component; III, authentic cholesterol plus spot C component.

of authentic cholesterol, suggesting that the major active component in FCS ex. is cholesterol.

Effects of cholesterol or LDL on the induction of the primary antibody response under serum-free culture conditions

When authentic cholesterol was added to

Fig. 5. Mass Spectrometric Analysis of Spot C Component.

A) authentic cholesterol; B) spot C component.

Fig. 6. Augmentation of the Antibody Response by Cholesterol or LDL in BSF Medium.

BALB/c spleen cells were cultured with SRBC in RPMI-1640 medium containing 1% FCS and 250 $\mu\text{g}/\text{ml}$ β -CD or in BSF medium. Where indicated, FCS ex., LDL, or cholesterol was added to BSF medium. The data were presented as the mean number of PFC $\pm$ standard error from triplicate experiments.

BSF medium, the antibody response was augmented at 0.5~1.0 $\mu\text{g}/\text{ml}$ almost as efficiently as FCS ex. (Fig. 6). In this experiment, FCS ex. was added to the culture medium at the amount expected to be equivalent to that contained in 1% FCS. It was confirmed that this level of FCS ex. showed an optimum enhancing effect on the antibody response (data not shown). It has been reported that FCS contains approximately 500 $\mu\text{g}/\text{ml}$ cholesterol as lipoproteins.¹⁶⁾ This corresponds to about 5 $\mu\text{g}/\text{ml}$ when FCS is added to the culture medium at 1%. Since cholesterol is

Fig. 7. Augmentation of the Antibody Response by L-Alanine, Putrescine and Pyruvate in BSF Medium Supplemented with LDL or Cholesterol.

BALB/c spleen cells were cultured with SRBC in BSF medium or in 10% FCS-containing medium for 5 days. Where indicated, LDL (10 μ g/ml), cholesterol (Chol) (1 μ g/ml), putrescine (Put) (0.1 μ g/ml), L-alanine (Ala) (10 μ g/ml), or pyruvate (Pyr) (100 μ g/ml) was added to BSF medium. The data were presented as the mean number of PFC from duplicate cultures. The experimental variations between duplicate cultures did not exceed 15% of the mean values.

practically insoluble in water, free cholesterol might not be used efficiently by the lymphocytes. Thus, we tested the effects of LDL, an endogenous cholesterol carrier,¹⁷⁾ and found that LDL enhanced the antibody response a little more efficiently than free cholesterol, as shown in Fig. 6. The requirements for lipids and lipoproteins in the *in vitro* antibody response have been suggested by the experiments using delipidated FCS.¹⁶⁾ The magnitude of the response in cholesterol- or LDL-containing BSF medium was always lower than that in FCS-containing medium, suggesting that some additional factors contained in FCS are required for eliciting an optimal response. Based on our preliminary observations that the water-soluble fraction of FCS with the molecular weight less than 1000 also showed augmenting activities, we have screened various water-soluble compounds including amino acids, vitamins, nucleic acids, and polyamines. As shown in Fig. 7, we found that a comparable antibody response to that observed in FCS-containing medium was attained in LDL-supplemented BSF medium when L-alanine, pyruvate, and putrescine, were added. In the presence of cholesterol instead of

LDL, the response, however, was always lower under the same culture conditions. Polyamines have been reported to suppress proliferative responses of murine lymphocytes in FCS-containing culture medium.¹⁸⁾ Our data represent a new finding that exogenously added putrescine had positive effects on the generation of antibody-forming cells in serum-free culture. Although data were not presented, phosphatidylcholine, triacylglycerol (triolein), or unsaturated fatty acids (oleic acid and linoleic acid) complexed with fatty acid-free BSA were inactive as lipid sources either alone or in combination in our experimental systems. These results suggest that LDL, in which the cholesterol moiety would be important, is a most effective lipid source in the generation of antibody-forming cells.

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