COMPLETE REPLACEMENT OF SERUM BY ALBUMIN, TRANSFERRIN, AND SOYBEAN LIPID IN CULTURES OF LIPOPOLYSACCHARIDE-REACTIVE B LYMPHOCYTES

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Murine B lymphocytes can grow and mature to IgM and IgG secretion in cultures which contain, as requisites, a thiol such as β-mercaptoethanol (1) or α-thioglycerol (2), a growth-inducing mitogen such as bacterial lipopolysaccharide (LPS)[1] (3–6) and a growth-supporting serum, commonly fetal calf serum (4). The frequency of normal B lymphocytes which grow under these conditions can be increased by the addition of thymus cells to the cultures (7, 8). One of three splenic B cells will then initiate growth in concentrations as low as one reactive B cell per culture.

When B lymphocytes are incubated without serum in conventional culture medium, there is maturation to IgM secretion, providing the cell concentration is high (10^6 cells/ml or greater). However, even at high cell densities, there is no maturation to IgG secretion and very little cell growth (3, 9). The addition of serum allows growth and maturation of B and T cells at lower cell concentrations. This shows that lymphocytes require substances for growth and maturation which are not included in conventional tissue culture media. However, the use of serum entails the serious disadvantage of introducing an unknown and uncontrolled set of substances which influence "in vitro" growth and maturation of lymphocytes. This not only creates problems of reproducibility, but also makes it difficult to analyze these additional growth requirements of lymphocytes and to isolate and interpret the significance of biological activities released by the cells into the culture medium.

There is, therefore, a need for tissue culture media in which serum is replaced by purified active serum components. Recently, the role of serum in supporting the growth and differentiation of hemopoietic cells in culture has been substantially clarified (10). Most of the serum required for clonal growth of erythrocyte and macrophage precursors in culture can be replaced by a combination of four normal serum components: albumin, transferrin, lecithin, and selenium.

In this paper these observations are extended to the growth and maturation of B lymphocytes. We first introduce the use of a new medium formulation for lymphocyte culture. The new medium (M-DMEM) is an enriched modification of Dulbecco's modified Eagle's medium containing additional amino acids and

[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; FCS, fetal calf serum; IU, international units; LPS, lipopolysaccharide; M-DMEM, modified-Dulbecco's modified Eagle's medium; PFC, plaque-forming cell.

vitamins. We demonstrate that in B-cell cultures established in the richer medium, serum is totally replaced by albumin, transferrin, and soybean lipid. The serum-free culture conditions allow extensive growth, maturation to IgM and IgG secretion, and cloning of one in three splenic B cells in the presence of thymus cells down to limiting concentrations of responsive cells.

Materials and Methods

Animals. C57BL/6J mice, 6–16 wk of age, and Lewis strain rats, 4 wk of age, were obtained from the Institut für Biologisch-Medizinische Forschung AG, Füllinsdorf, Switzerland.

Cells. Mouse spleen cells were dispersed in a phosphate-buffered salt solution either by gentle teasing and pipetting or by passage through a 200 mesh stainless steel screen. After two washings they were resuspended in tissue culture medium. Rat thymus cells were similarly dispersed through a steel screen and washed. Small, resting splenic lymphocytes were obtained by velocity sedimentation of spleen cells at unit gravity (11, 12).

Medium. RPMI 1640 medium was obtained complete from Microbiological Associates (Walkersville, Md.). Before use, it was supplemented with 2 mM glutamine, 10 mM Hepes buffer (pH 7.3, Grand Island Biological Co., Grand Island, N.Y.), 100 international units (IU)/ml penicillin, 100 μgm/ml streptomycin, and either 5×10^{-5} M β-mercaptoethanol or 7.5×10^{-5} M α-thioglycerol.

M-DMEM was prepared by addition of the following to GIBCO's Dulbecco's powdered medium formulation H-21: L-alanine (25 mg/liter), L-asparagine (25 mg/liter), L-aspartic acid (30 mg/liter), L-cystine (22 mg/liter, to obtain a total of 70 mg/liter), L-glutamic acid (75 mg/liter), L-proline (40 mg/liter), Na pyruvate (110 mg/liter), vitamin B_{12} (0.013 mg/liter), biotin (0.013 mg/liter), Na selenite (Na_2SeO_3, 0.0173 mg/liter), all essentially as described earlier (9). Correction of the final osmolarity of the medium required addition of 200 ml water per liter of medium. (The osmolarity was considered "correct" when cultures tolerated the addition of 15 mM NaCl, or the equivalent dilution with water, with no adverse effect. The optimum was 280 mOsM as measured by freezing point depression). Alternatively, the correct osmolarity was achieved by using a specially modified Dulbecco's H-21 powder formula prepared for us by Grand Island Biological Co. containing all of these supplements but with NaCl reduced to 4505, KCl to 330, and $CaCl_2$ to 165 mg/liter. In either case, further additions, with final concentrations indicated, were α-thioglycerol (7.5×10^{-5} M), Hepes buffer (25 mM, pH 7.3), $NaHCO_3$ (30 mM), penicillin (100 IU/ml), and streptomycin (100 μg/ml). There was no attempt to adjust the pH. The complete medium was filter-sterilized, overlaid with CO_2, and stored in aliquots at −20°C in the dark until use.

Pure human transferrin (Behringwerke, Marburg/Lahn, W. Germany) was dissolved in M-DMEM (90 mg/ml). It was 1/3 Fe saturated by addition of 7.4×10^{-9} mol of Fe^{+++} per mg transferrin in the form of $FeCl_3$ dissolved in 0.1 mM HCl. The stock was stored at 4°C after filter sterilization. Purified bovine serum albumin (BSA) (Behringwerke) was dissolved in water, 5 g/100 ml. It was delipidated (10, 13, 14) as follows: the pH of the solution was adjusted to 3.0 with concentrated HCl. Dextran T 40 (Pharmacia, Uppsala, Sweden) (1 mg/ml) and activated charcoal (Norit SX-1, 10 mg/ml, Norit, N.V., Amersfoort, Holland) were added and the mixture was incubated at 56°C for 30 min with frequent gentle agitation. After removal of the charcoal by centrifugation and Millipore filtration, the pH was brought to 6.5 with 2 M NaOH. The solution was deionized by incubation without agitation at 4°C overnight with 1.5 cm^3 Amberlite mixed bed ion exchange resin MB-1 (Serva, Heidelberg, W. Germany) per gram BSA. After four times concentration over an Amicon Diaflo UM-10 membrane, (Amicon Corp., Scientific Sys. Div., Lexington, Mass.) the solution was filter sterilized and stored at 4°C.

Further purification of transferrin or delipidated BSA (40 mg/ml) was carried out by passage through a column of Sephadex G-150 in 0.1 M NaCl, 0.05 M Tris HCl pH 7.4 (2.5 × 90 cm, applying 3- to 5-ml loads). Toxicity in the BSA observed after chromatography was removed by deionization over Amberlite MB-1.

Soybean lipid (200 mg) (soybean lecithin, PH 75, Nattermann & Cie., Cologne, W. Germany), and cholesterol (50 mg) were completely dispersed in 50 ml acid medium (see below) by ultrasonication at maximum amplitude for 1 h at 0°C in air using a Measuring and Scientific Equipment, Ltd. sonicator with a 2-cm diameter titanium probe. The suspension was then

sterilized by passage successively through 1.2 and 0.45 μm Millipore filters. The acid medium was a solution of GIBCO's Dulbecco's H-21 powder containing no bicarbonate or other additives except for 1% delipidated BSA and antibiotics. Its pH was 5.1. The presence of BSA during sonication was important to both the activity and the stability of the preparation. The suspension was stored at 4°C and no deterioration in activity was observed over several weeks. A single batch of fetal calf serum (FCS) (Grand Island Biological Co. batch K255701D) was used where indicated.

Cells were cultured in 0.2-ml volumes either in loosely capped 5-ml plastic tubes (no. 2058, Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) or in Micro Test II plates (no. 3040, Falcon Plastics). Incubation was at 37°C in a humidified atmosphere containing 5% CO_2.

LPS. Purified LPS-S from *S. abortus equii* was provided by Doctors C. Galanos and O. Lüderitz (Max-Planck Institut für Immunobiologie, Freiburg, W. Germany). It was dispersed in buffered saline and used in culture at 10–50 μg/ml as described earlier (15).

Detection of Ig-Secreting Cells. All cells secreting a particular Ig class can be detected, regardless of their antigen specificity, by using a modification of Jerne's hemolytic plaque method. Briefly, Staphylococcal protein A is coupled to sheep erythrocytes. The protein A-coated erythrocytes are plated in agar with class-specific anti-mouse Ig antiserum, complement, and the secreting B cells. Complexes are formed between secreted Ig and the anti-Ig antibodies. Erythrocytes near Ig-secreting cells bind these complexes at their surface and are lysed by the action of complement. The experimental details are given elsewhere (16).

Results

Serum Dependence of LPS-Induced Generation of IgM-Secreting Cells in Either RPMI 1640 or M-DMEM. Low concentrations of mouse spleen cells, i.e., 3×10^5 cells/ml were cultured in the presence of LPS. The number of effector B cells induced by LPS was assessed after 5 days, by using the protein A-sheep erythrocyte hemolytic plaque assay which detects all IgM-secreting cells. The results (Table I, lines 1 to 4) indicate that in either medium, the generation of IgM-secreting plaque-forming cells (PFC) depended on the addition of serum at these low cell concentrations. With 5% FCS, responses in M-DMEM equalled and often exceeded those obtained with the older conditions.

Replacement of Serum by Albumin, Transferrin, Soybean Lipid, and Cholesterol. The effects of albumin, transferrin, crude soybean lipid, and cholesterol are also shown in Table I. Their addition in combination to cultures containing serum often had a small supportive effect (lines 5 and 7 compared with 1 and 3). There was also a slight effect when they were added to cultures in serum-free RPMI 1640 (line 6 compared with 2). However, when added to serum-free M-DMEM, their effect was dramatic: PFC developed in numbers equivalent to those obtained with serum (line 8). The effects of albumin, transferrin, and soybean lipid were demonstrated individually by omitting them singly from the mixture (lines 9–12). Deletion of any one greatly reduced the number of PFC formed, with the exception of cholesterol whose deletion alone consistently had little effect. As in serum-containing cultures, the requirement for mitogen (here, LPS) remained (line 13).

The reproducibility of the LPS response in serum-free conditions is indicated in Table II. The table summarizes the results of nine consecutive experiments with M-DMEM. On average, albumin, transferrin, soybean lipid, and cholesterol completely replaced serum in M-DMEM, and gave LPS responses more than twofold higher than those obtained routinely with supplemented RPMI-1640 and serum.

Experiments were performed to determine optimum concentrations of these

TABLE I

Replacement of Serum by BSA, Transferrin (Tf), Soybean Lipid, and Cholesterol (chol)

	FCS	LPS	BSA	Tf	Soybean lipid	chol	PFC*
RPMI 1640							
1.	+	+	–	–	–	–	11,750
2.	–	+	–	–	–	–	667
M-DMEM							
3.	+	+	–	–	–	–	23,500
4.	–	+	–	–	–	–	1,833
RPMI 1640							
5.	+	+	+	+	+	+	17,583
6.	–	+	+	+	+	+	1,250
M-DMEM							
7.	+	+	+	+	+	+	38,111
8.	–	+	+	+	+	+	34,667
9.	–	+	–	+	+	+	4,167
10.	–	+	+	–	+	+	83
11.	–	+	+	+	–	–	3,125
12.	–	+	+	+	+	–	44,917
13.	–	–	+	+	+	+	83
14.	–	+	+	+	⊕	+	917

* Number of IgM PFC induced by LPS after 5 days of culture, expressed per 10^5 cells initially plated.

‡ Chromatographically pure soybean lecithin (P-L Biochemicals Inc., Milwaukee, Wis.)

TABLE II

LPS Responses in Serum-Containing and Serum-Free Conditions. Cultures were Initiated with 3 × 10^5 Spleen Cells/ml, and Results are Expressed as IgM PFC Induced Per 10^5 Cells Originally Plated

	RPMI 1640 5% FCS – – – –*	M-DMEM 5% FCS – – – –	M-DMEM 5% FCS + + + +‡	M-DMEM 0 FCS + + + +
Number of experiments	9	9	9	9
Mean of IgM-PFC/10^5 cultured spleen cells	12,000	21,600	32,900	35,100
Standard error of mean	1,570	2,820	4,270	5,250
Relative error	0.13	0.13	0.13	0.15

* Without albumin, transferrin, soybean lipid, and cholesterol.

‡ With albumin, transferrin, soybean lipid, and cholesterol.

substituents. The optimum concentration of albumin was 400 μg/ml (6×10^{-6} M), of which 275 μg/ml were added lipid free, and 175 μg/ml in association with soybean lipid. Soybean lipid had its maximum effect at 50–100 μg/ml assuming that all of the lipid was completely dispersed. The transferrin effect was maximum at 1 μg/ml (1×10^{-8} M) with no toxicity evident beyond 360 μg/ml.

Partial Purification and Characterization of the Active Serum Substituents. In most of our experiments, albumin, transferrin, and soybean lipids were used as supplied commercially, except that the albumin was routinely delipidated and deionized. However, as a test of whether their activities might reflect the presence of active contaminants, they were subjected to additional purification before being used in the experiment shown in Table I. Albumin and transferrin were subjected to gel permeation chromatography on Sephadex G-150. The protein peaks of both the albumin and the transferrin preparations were eluted at positions corresponding to their monomeric molecular weights. Narrow cuts at the monomer peaks were taken for use in culture. Electrophoresis on SDS-polyacrylamide gels (pH 6.8, 5% β-mercaptoethanol) revealed faint Coomassie Blue stainable contaminants in the albumin but not the transferrin preparations. The impurities were visible only when the gels were loaded with 6 or more μg of protein. Since the threshold of detection in the system used is about 10–50 ng (17), the contaminants probably did not exceed 1–2% of the albumin. Gel filtration removed those impurities with molecular weights higher than albumin but not those of lower molecular weight. Dose-response experiments indicated no change in effectiveness of the transferrin or albumin after gel filtration.

The soybean lipid was a crude lecithin preparation containing approximately 40% triglyceride, 60% phosphatide (phosphatidyl choline ("lecithin"), phosphatidyl ethanolamine, and monophosphatidyl inositol), and traces of sterol. When the preparation was repeatedly washed with acetone to remove lipids other than phosphatides, the activity of the remaining material was considerably reduced. A commercially supplied chromatographically pure soybean lecithin preparation was actually inhibitory (Table I, line 14) at doses comparable to those at which the crude product was active, and had no effect at lower doses. It therefore appears that lipids other than lecithin in the crude preparation are important to its activity or stability.

Survival of LPS-Reactive Splenic Lymphocytes in M-DMEM Containing Either Fetal Calf Serum or Albumin, Transferrin, Soybean Lipid, and Cholesterol. Survival of LPS reactivity of small resting B lymphocytes obtained by velocity sedimentation of spleen cells, was tested in M-DMEM containing either fetal calf serum or the serum substituents. Three times 10^5 small splenic lymphocytes were put in culture and LPS was added either at the beginning or after a variable delay. Cells were counted each day. The number of cells initiating growth on addition of LPS was estimated by extrapolating the exponential portion of each growth curve to the time of LPS addition. The results in Fig. 1 show that, in the presence of serum, LPS reactivity of the small spleen cells was lost rapidly with a half time of about 6 h. In contrast, LPS reactivity was unchanged for 2 days in serum-free medium containing only albumin, transferrin, soybean lipid, and cholesterol, and then declined with a half time of about 36 h. The overall survival of small spleen cells cultured in the absence of LPS was also prolonged. In medium containing serum, the half life of the cells was 10–12 h, while in serum-free medium containing only the serum substituents, survival half time increased to about 40 h. Addition of fetal calf serum (10%) to M-DMEM containing the four substituents did not alter the survival times of LPS reactivity or of total cells. We conclude that albumin,

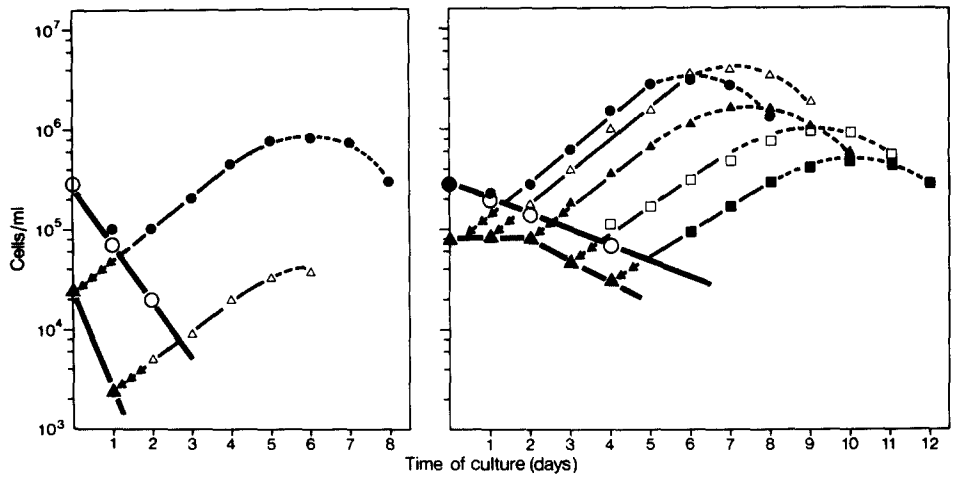

FIG. 1. Survival of lymphocytes (○) and of LPS reactivity of splenic B lymphocytes (▲) in M-DMEM containing FCS (left) and in M-DMEM containing albumin, transferrin, soybean lipid, and cholesterol (right). Growth-inducing mitogen (LPS) was added either at day 0 (●), day 1 (△), day 2 (▲), day 3 (□), or day 4 (■) of culture. Cells in culture were counted each day. The number of LPS-reactive B cells (▲) was determined by extrapolation (◀◀◀◀) of the exponential parts of the growth curves to the time of LPS addition. Survival of splenic lymphocytes was determined in cultures without mitogen.

transferrin, soybean lipid, and cholesterol increase the survival times of both LPS-responsive B cells and of total cells.

Growth and Maturation of Every Growth-Inducible B Cell at Limiting Dilution in the Absence of Serum. The addition of syngeneic or xenogeneic thymus cells to cultures of LPS-reactive B cells increases the frequency of growth-initiating cells and allows their growth even when diluted to one LPS-reactive B cell per culture (7, 8). These diluted cultures also normally require serum. We therefore investigated whether albumin, transferrin, soybean lipid, and cholesterol could substitute for serum in cultures at limiting dilutions of reactive B cells. Splenic lymphocytes were serially diluted in M-DMEM containing thymus cells and either serum or the four serum substituents, down to concentrations limiting the number of LPS-reactive B cells to one per culture. IgM-secreting PFC were enumerated after 5 days of culture.

The results in Table III show that serum can be completely replaced in these cultures by the serum substituents. With or without serum, one of six spleen cells reacted to LPS by generating a clone of IgM-secreting PFC. (According to Poisson's distribution, an average of one reactive cell is present per culture when 37% of all cultures do not show a response.) The four substituents did not replace the growth-supporting effect of the thymus cells: in the absence of thymus cells, B-cell cultures ceased to respond to LPS below 3×10^4 cells/ml in medium containing only fetal calf serum, and at 8×10^3 cells/ml in serum-free medium containing the serum substituents.

LPS-Induced IgG Synthesis and Secretion. LPS induces the growth and maturation of clones which secrete IgG (4). The frequency of LPS-reactive precursors yielding an IgG-secreting clone is about $^1/_{10}$ that of precursors for

TABLE III

Frequencies of LPS-Reactive B Cells Yielding Clones of IgM Secreting PFC after 5 Days in Serum-Containing and Serum-Free Cultures

Spleen cells per 0.2 ml culture	IgM PFC in medium containing											
	10% FCS						BSA, Tf, and lipid					
	No thymus cells			Plus thymus cells			No thymus cells			Plus thymus cells		
	Mean PFC*	Range	Percent negative cultures	Mean PFC*	Range	Percent negative cultures	Mean PFC*	Range	Percent negative cultures	Mean PFC*	Range	Percent negative cultures
6×10^4	101	15–350	0	over 1,000	–	0	185	25–550	0	over 1,000	–	0
6×10^3	14.3	8–25	85	650	165–over 2,000	0	29.2	10–65	35	700	150–over 1,000	0
6×10^2	–	–	100	380	55–over 1,000	0	–	–	100	420	60–over 1,000	0
6×10^1	–	–	100	113	10–400	5	–	–	100	118	25–350	5
6×10^0	–	–	100	34.3	8–156	35	–	–	100	30.2	15–54	34

* Per positive 0.2 ml culture.

TABLE IV

Frequencies of LPS-Reactive B Cells Yielding IgG-Secreting PFC in 60 Spleen Cells Cultured 7 days in the Presence of Thymus Cells with and without Serum

Medium containing	Mean PFC per positive culture	Range	Percent negative cultures
20% Fetal calf serum	74.1	11–221	28
BSA, TF, and lipid	68.7	15–241	31

IgM secreting clones.[2] Normally, the induction of IgG-secreting B cells is strongly serum dependent and is optimal only at 20% serum. We therefore tested whether serum could also be replaced for induction of IgG secretion by LPS. IgG-secreting cells were assayed at day 7 of culture when their number was found to reach its maximum. The frequency of LPS-reactive precursors of IgG secreting cells was determined by serially diluting the cells into cultures containing M-DMEM, LPS, and thymus cells. The results in Table IV show that 20% fetal calf serum was totally replaced by the serum substituents for the induction of IgG-secreting cells in culture. The frequency of precursors yielding clones of IgG-PFC was 1 of 60 spleen cells in both conditions of culture, and there was no difference in the average size of the clones generated.

Discussion

The present results remove much of the mystery surrounding the serum requirement in lymphocyte cultures. With the important reservation that our materials were not entirely free of impurities, it appears that the role of serum is confined mainly and perhaps entirely to supplying transferrin, lipid, and albumin to the cells.

Transferrin is the major iron-carrying protein of serum, normally present at

[2] J. Andersson, A. Coutinho, and F. Melchers. Manuscript in preparation.

a concentration of 3–4 mg/ml (18). Until now, much of the interest in it has centered on its role in supplying erythrocyte precursors with iron for hemoglobin synthesis. However, reports have appeared showing effects on growth of other cells including PHA-responsive lymphocytes (19), spleen cells (20), granulocyte/macrophage precursors (10), pituitary cells (21), and fibroblasts (21). The present results extend the list to include LPS-responsive B lymphocytes. Whether these requirements reflect simply a requirement for iron by growing cells, or whether transferrin may have some other as yet unidentified function, remains an intriguing question. However, the trivial possibility remains to be excluded that it exerts its effect in culture simply by binding toxic metal ions.

That lipids can be growth-supporting for cells in serum-free culture has long been recognized (22). However, their inclusion in medium is made complicated by their insolubility in water. In this study, lipids were introduced into the medium in the form of sonicated dispersions of soybean phospholipid, soybean triglyceride, and cholesterol. Sonication of phospholipid-containing mixtures results in the formation of lipid bilayers arranged in the form of vesicles or "liposomes" (see 23 for a comprehensive treatment of this subject). The lipid could become available to cells by fusion of the vesicles with cell surface membranes or by phagocytosis of the vesicles (23). Other possible modes of lipid delivery could involve albumin, which is capable of binding lecithin in addition to other lipids (24). The lipid dispersions were most active when sonicated in the presence of albumin. Albumin does not associate with the vesicles under these conditions (25). However, at the low pH used, some of the soybean lipid may leave the vesicles to complex with albumin (25). The soybean lipid was a mixture of phosphatides and triglycerides of heterogeneous fatty acyl composition. While triglyceride-free soybean lecithin was not active by itself, it is not clear whether the triglycerides might be active alone, or whether the cells need both phosphatide and triglyceride together, or whether the triglycerides might serve simply to stabilize or inhibit oxidation of the phospholipids. However these questions are resolved, it is clear that the serum-free conditions will provide fertile ground for investigation of lipid requirements and roles in these cells.

No cholesterol requirement could be demonstrated in these conditions of culture. However, it has been included routinely in our experiments on the basis of the requirement for it in serum-free cultures of erythrocyte precursors (L. J. Guilbert, unpublished observations) as well as for colony formation by B lymphocytes in serum-free methyl cellulose cultures (N. N. Iscove, unpublished observations), and because lecithin dispersions prepared without cholesterol tend to deplete cell membranes of cholesterol (26).

A requirement for albumin by concanavalin A- (27) and PHA-reactive (28) human lymphocytes has been shown before. Albumin can complex with a wide variety of molecules, many by hydrophobic interaction. It has been suggested that one function in culture could be to bind components present in the medium in inhibitory concentrations (10). In addition, our results as well as those of others (28) suggest that albumin plays a role in making lipids available to the cells, a function also provided in vivo by the serum lipoproteins.

Hemopoietic cells cultured in serum-free medium show a requirement for selenium (10), as do freshly explanted fibroblasts (29). A similar requirement

was not apparent in the present study, perhaps because sufficient selenium was carried over into the cultures by the cells themselves. Since such a need could arise in other culture conditions, selenium has been included as part of the modified Dulbecco's medium.

We wish to stress that the inclusion of albumin, transferrin, and lipids alone was not sufficient for adequate growth support in RPMI 1640 in the absence of serum. The results presented here also depended on the use of M-DMEM, a richer medium formulation than the more conventional RPMI 1640. Compared to RPMI 1640, the new medium contains two to three times higher concentrations of amino acids and fourfold higher vitamin concentrations. In addition, it contains substances not present in RPMI-1640, including pyruvate, L-alanine, and selenium. The relative contributions of these added ingredients and the numerous quantitative differences have not been investigated in detail.

Even in the improved medium and with the addition of the serum substituents, the efficiency of the LPS response decreased with decreasing cell numbers per culture. The addition of thymus "filler" cells (7) therefore remained essential for a high frequency of growth initiation at sparse seeding densities. The nature of the requirements supplied by the filler cells remains unknown. However, it is conceivable that even these requirements are "nutritional" in nature and may eventually turn out to be replaceable by defined substances.

It is worth emphasizing that the induction of resting B cells to growth and maturation to immunoglobulin secretion in the improved conditions also continued to depend on the presence of LPS (4). This requirement appears analogous to that for erythropoietin by erythrocyte precursors and for colony-stimulating factor by macrophage precursors. The generalization appears to hold that, in addition to a common set of "nutritional" requirements, the growth of normal cells requires interactions with specific effector substances, each acting on a particular class of differentiated cell. LPS appears to fulfill such an effector (or "mitogenic") role for a subset of B lymphocytes in culture, and is not replaced by any other component of the medium described. The fact that responses were as high in serum-free conditions as they were in serum-containing cultures suggests that none of the hormones normally present in serum are required for B-lymphocyte growth. Any steroid hormones originally carried by the albumin would have been greatly reduced, if not eliminated entirely, by the charcoal extraction procedure (14, 30).

The conditions developed here have been successfully applied without modification to other lymphocyte systems (A. Coutinho, F. Melchers, W. Lernhardt, and G. Heinrich, unpublished observations). These include concanavalin A-reactive T cells and mixed lymphocyte reactions, with net growth and development of killer cells in both, and growth and cloning of transformed T- and B-cell lines previously growing in fetal calf or horse serum. No period of adaptation to serum-free medium was necessary for these lines; commencement of growth was immediate. Myeloma cells grown to exhaustion in the new conditions have yielded two to fivefold more secreted Ig than was previously attainable.

It can be expected that these conditions will have wide applicability to the study of immune phenomena in vitro. The improved survival of cells in the new conditions will enlarge the scope of feasible experimentation in vitro and should allow more extensive expansion of clones derived from explanted normal cells.

The potential advantages of reproducibility and ease of isolation and assay of active cell products in defined conditions of culture need no elaboration.

Summary

Albumin, transferrin, and lipids can replace serum entirely for support of LPS-stimulated murine B lymphocytes in culture. In the presence of these compounds, growth and maturation to IgM and IgG secretion, induced by lipopolysaccharide (LPS), occurs at the same or higher efficiency in serum-free conditions as in conventional serum-containing medium, even at relatively low cell concentrations. In contrast to the rapid disappearance of LPS reactivity in conventional serum-containing medium, responsiveness remains at initial levels in serum-free conditions for 2 days before slowly declining. Overall lymphocyte survival is also markedly prolonged. In the presence of thymus "filler" cells, the serum-free conditions permit growth of every LPS-responsive cell to a clone of Ig-secreting cells at dilutions as low as a single reactive B cell per culture. The results have several important implications. These include the establishment for the first time of transferrin as a requirement for B lymphocyte responses in culture, and the availability now of conditions for the assay and isolation of cell products regulating lymphocyte function, free of interference from undefined serum components.

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