

Development of a chemically defined serum- and protein-free medium for growth of human peripheral lymphocytes

(immunology/mitogen/proliferation/blastogenesis)

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Communicated by Esmond E. Snell, August 12, 1985

ABSTRACT A chemically defined, protein-free medium (designated CFBI 1000, where CFBI = Clayton Foundation Biochemical Institute) that supports human peripheral lymphocyte proliferation has been developed. This medium allows exploration of individual metabolic differences by varying the medium composition as well as providing a base to explore further the mechanisms of lymphocyte activation in a system initially free of added macromolecular species other than mitogen. The peripheral blood lymphocyte is an ideal system for metabolic studies because it is easily obtained, is a primary resting cell that can be activated to proliferate, and presumably reflects both the genetic makeup and biochemical environmental history of the individual at the time the cells were formed. Examination of the role of various factors in lymphocyte activation and subsequent events may be simplified by the utilization of a medium that is protein-free and chemically defined. The CFBI 1000 medium supports the growth response of human peripheral lymphocytes to mitogen as measured by [³H]thymidine incorporation to an extent comparable to other media used widely in assessment of lymphocyte proliferation.

The effects of alterations in medium upon growth of cells taken directly from an individual subject have been investigated for a number of years in this laboratory as an approach to the individualized assessment of metabolic and nutritional status. The ready availability of lymphocytes from human peripheral blood relative to other cells and their known activation and blastogenesis in culture media containing fetal calf serum led to their selection as potential cells for this work (1, 2). The primary resting state of the T lymphocyte can be interrupted by the presence of antigen in association with major histocompatibility complex or, more commonly in cell culture systems, a mitogen such as phytohemagglutinin (PHA) or Con A (1). The transition from the resting state to proliferation of lymphocyte cells presumably involves the multiple enzymatic processes characteristic of any growing cell; consequently, it would be anticipated that abnormalities or anomalies in nutrient requirements and metabolism of the lymphocytes would reflect conditions existing in cells of other tissues as well. Furthermore, the metabolic condition of lymphocytes may be influenced by biochemical parameters at the time of their formation and may thereby provide information on past cellular environment (3, 4).

The association of a clinically recognizable disease with deficiencies of a specific nutrient has led, in most instances, to the rapid use of supplements of that nutrient in the medical treatment of the disease (3). However, deficiencies of many nutrients—e.g., pyridoxine or riboflavin—do not result in a single clinically recognizable disease but rather in a number of different symptoms manifested in different individuals (3).

Considerable evidence has accumulated for a relationship between metabolic (and nutritional) status and disease states in individuals (3); however, the incorporation of such concepts into general clinical practice has not occurred, largely because broadly based methods for assessment of individual metabolic and nutritional status are not presently available. To provide an adequate assessment of individual requirements, metabolic function must be assessed over a wide range of reactions rather than simply determining the serum or excretion concentrations of the individual nutrients or the degree of coenzyme saturation of a single enzyme.

We report here a simple, easily manipulated, protein-free medium (designated CFBI 1000, where CFBI = Clayton Foundation Biochemical Institute), whose components have been optimized to support the growth of mitogen-activated, human peripheral blood lymphocytes. The components of Ham's MCDB 104 medium without added serum or protein were used as the starting point for development of a medium to support lymphocyte proliferation (5). The techniques used in the development of this medium were similar to those described by Ham (6) except that minimal concentrations for optimal responses were determined and used in the development of the medium as described previously (2). This chemically defined and protein-free medium allows the direct culture of lymphocytes with growth responses comparable to those obtained with serum-supplemented media.

MATERIALS AND METHODS

Materials. The components for CFBI 1000 medium were purchased from the sources listed in Table 1. Histopaque 1077 (Ficoll/sodium diatrizoate, type 400) and PHA (L-8754) were purchased from Sigma. Aphidicolin was obtained from the Natural Products Branch, Division of Cancer Treatment, National Cancer Institute. Fetal calf serum and premixed antibiotic solution were obtained from GIBCO. Culture tubes (12 × 75 mm) were purchased from Scientific Products or Fisher (manufactured by Elkay), from Van Waters and Rogers (manufactured by Labconco), or from Falcon. Growth of lymphocytes can be influenced by subtle differences in the tube-manufacturing process, with almost total inhibition from unknown causes in some lots of tubes. Disposable plastic pipettes were obtained from Falcon. All other tissue culture plastic ware (centrifuge tubes, microtiter plates) were from Corning. [³H]Thymidine (specific activity, 6.7 Ci/mmol; 1 Ci = 37 GBq) was purchased from ICN, and 2,5-diphenyl-1,3-oxazole was purchased from Beckman. Scintillation-grade toluene was obtained from Baker. All other chemicals were reagent-grade. Filters (0.2 μm) for sterilization were from Nalgene or Millipore.

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Abbreviation: PHA, phytohemagglutinin.

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Medium Composition and Preparation. The composition of CFBI 1000 medium is presented in Table 1. Tissue culture-grade water for CFBI 1000 medium is prepared from water distilled in a metal still and subsequently passed through a Continental Water Systems activated carbon cartridge and then through a mixed-bed resin (40% Rohm-Haas IR 120; 60% Rohm-Haas IRA-410 ion-exchangers). Alternately, water was passed through a Nanopure system (Barnstead) with two activated carbon filters, a mixed-bed resin, and a micron filter and was subsequently distilled from glass (Bellco Glass). Deionized, distilled water was heated to boiling and then degassed under a vacuum; this final procedure removes any residual chlorine, which is detrimental to lymphocyte growth in this medium. Conductivity of the water was $<1 \mu\text{S}$. All solutions for CFBI 1000 medium are made by using this tissue culture-grade water.

The preparation of the medium is described in detail, as the formulation is sensitive to the order of addition of the various component chemicals. CFBI 1000 medium is prepared from solid materials and a series of sterile stock solutions, which can be sterilized by filtration and stored in aliquots at -20°C (except phenol red, MgSO_4 , and CaCl_2 stock solutions are stored at 25°C). The medium (1 liter) is prepared as follows. Stock solution IA (500 ml) is formulated by dissolving 11.9 g of Hepes, 0.525 g of K_2HPO_4 (anhydrous), and 7.01 g of NaCl in 400 ml of tissue culture-grade water. Phenol red (1 ml, 1.24 g/liter) is added and the pH of the solution is adjusted to 7.6 with the required volume of NaOH. All solutions added subsequently must be at approximately neutral pH. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (5 ml, 49.3 g/liter), 10 ml of a solution of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.278 g/liter) and Na_2EDTA (0.149 g/liter), and 5 ml of antibiotic solution (GIBCO; penicillin, 10,000 units/ml; streptomycin, 10,000 $\mu\text{g}/\text{ml}$; and Fungizone, 25 $\mu\text{g}/\text{ml}$) are

added with stirring; the final volume is adjusted to 500 ml with tissue culture-grade water. This solution is sterilized by filtration. Filtered stock solution IA is stable for at least 4 weeks when stored at 4°C . To this mixture glucose (720 mg) is added, followed by addition of 10 ml each of the following stock solutions, which contain 100-fold the medium concentrations: (i) amino acids (except cysteine and glutamine), (ii) glutamine, (iii) sodium pyruvate and cysteine, (iv) *myo*-inositol and choline chloride, (v) vitamins, and (vi) adenine. Concentrated vitamin solutions are used to make up the 100-fold stock solution. CaCl_2 (5 ml, 22.2 g/liter) is added last to prevent precipitation of the Ca^{2+} . The final volume of the solution is brought to 1 liter with tissue culture-grade water, and the medium is sterilized by filtration. Mitogen (2 ml, 1 mg of PHA per ml) is added after sterilization to prevent any protein loss on the filter. CFBI 1000 medium is stored at 4°C and is stable for at least 4 weeks at this temperature.

Washing Buffer. The buffer solution used in the isolation of lymphocytes from whole blood is prepared as follows. Stock solution IA without MgSO_4 , iron sulfate/EDTA, and antibiotic solution is diluted 1:1 with tissue culture-grade water. To 100 ml of this solution are added 0.25 ml each of the previously cited stock solutions of MgSO_4 , iron sulfate/EDTA, and CaCl_2 . The washing buffer is sterilized by filtration and stored at 4°C .

Isolation of Lymphocytes. Blood is collected by venipuncture in tubes (Venoject) containing solid sodium heparin. Human lymphocytes are isolated from this peripheral blood by the method of Boyum (7) or by the method of Ferrante and Thong (8). Briefly, either blood diluted (1:1.4, vol/vol) with phosphate-buffered saline or whole blood is layered onto Histopaque 1077 and centrifuged at $400 \times g$ for 20 min. The lymphocyte layers from these tubes are carefully removed

Table 1. Composition of CFBI 1000 medium

Compound	mg/liter	Compound	mg/liter
Amino acids		Other organic compounds	
L-Arginine	7.00	Adenine	1.35
L-Cysteine	26.4	Choline chloride	14.0
L-Glutamine	58.5	<i>myo</i> -Inositol	18.0
Glycine	2.50	D-Glucose	720.0
L-Histidine-HCl	2.30	Sodium pyruvate	165.0
L-Isoleucine	1.30	Sodium EDTA	1.49
L-Leucine	4.40	Inorganic salts	
L-Lysine	12.2	CaCl_2 (anhydrous)*†	111.0
L-Methionine	1.50	$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	2.78
L-Phenylalanine	0.83	K_2HPO_4 (anhydrous)‡	525.0
L-Serine	3.50	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ §	246.5
L-Threonine	4.00	NaCl	7010.0
L-Tryptophan	0.34	Buffer and indicator	
L-Tyrosine	0.64	Hepes	11,900.0
L-Valine	3.90	Phenol red	1.24
Vitamins		NaOH† to give pH 7.60¶ in air at 25°C	
Biotin	0.0074	Antibiotics	
Folic acid	0.000602	Penicillin at 50 units/ml	
Hydroxocobalamin	0.14	Streptomycin	50
Niacinamide	0.61	Fungizone	0.13
D-Pantothenic acid (hemicalcium salt)	0.24	Lectin	
Pyridoxine-HCl	0.062	PHA	2
Riboflavin	0.0038		
Thiamine-HCl	0.34		

All chemicals were obtained from Sigma unless otherwise designated.

*Or equivalent amount of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (Mallinckrodt).

†Baker.

‡Sigma or Baker.

§Mallinckrodt.

¶Final pH when equilibrated at 37°C with 5% CO_2 is 7.1.

||GIBCO.

with a sterile Pasteur pipet and transferred to a 15-ml sterile centrifuge tube containing washing buffer. The lymphocytes are washed twice with washing buffer, and the final cell pellet is resuspended in washing buffer (0.75 ml for each 10 ml of blood). The cell number is determined either by counting a 20- μ l sample in 10 ml of Isoton II with three drops of Zapoglobin on a model ZBI Coulter Counter (all from Coulter) or by counting a 50- μ l sample on a hemocytometer. The cell suspension is diluted with washing buffer to a final concentration of 3×10^6 cells per ml. CFBI 1000 medium is inoculated to a final cell concentration of 1.5×10^5 cells per ml in tubes (1 ml) or microtiter plates (0.2 ml). The cells are incubated for 4 days at 37°C in a 5% CO₂/95% air atmosphere; CO₂ levels are monitored frequently and readjusted as necessary. The incubator humidity is maintained near 100%, and baffles are used at the ends of shelves to prevent evaporation in microtiter plates due to air currents. After 4 days of incubation, 50 μ l of [³H]thymidine (5.9 μ M, specific activity, ≈ 1.0 mCi/ μ mol) is added per ml of medium, and the incubation is continued for 12–24 hr. The cells are harvested with a Brandel M-12 cell harvester (Brandel, Gaithersburg, MD) using Reeves-Angel 934 AH filter paper. Dried filters are placed in scintillation fluid containing 5 g of 2,5-diphenyl-1,3-oxazole per liter of toluene, and the [³H]thymidine retained on the filters is determined by assay in a Beckman LS250 scintillation counter.

Growth in RPMI Medium. RPMI 1640 medium (where RPMI = Roswell Park Memorial Institute) was purchased from Irvine Scientific and supplemented with 300 mg of L-glutamine per liter; antibiotic solution was added as for CFBI 1000 medium, and mitogen was added to sterilized medium. Alternately, RPMI 1640 medium was formulated in the laboratory with components common to CFBI 1000 medium from the same source. No significant differences in results were noted between commercial and laboratory-prepared RPMI 1640 media. Lymphocytes were inoculated into this medium as described for CFBI 1000 medium.

RESULTS

Growth of Lymphocytes in CFBI 1000 Medium. The growth of PHA-stimulated lymphocytes in CFBI 1000 medium as measured by [³H]thymidine incorporation is depicted in Fig. 1. Activation occurred within 24–36 hr, and lymphocyte proliferation reached a maximum at 5.5–6 days and then declined over the following period in culture. The decline in growth rate past the maximum was slightly lower in microtiter wells than in tubes. The individual responses in Fig. 1 are normalized to percent of the maximum uptake of thymidine, which averages about 20,000–30,000 cpm in the tubes.

Comparison of CFBI 1000 Medium with RPMI 1640 Medium. A medium commonly used for growth of human peripheral blood lymphocytes is RPMI 1640 supplemented with L-glutamine, bacterio- and fungicide and 5–20% heat-treated fetal calf serum. The abilities of the RPMI 1640 and CFBI 1000 media to support lymphocyte proliferation in the presence and absence of fetal calf serum were compared, and the results for several individuals are shown in Fig. 2. The amount of radiolabel incorporated into DNA in the CFBI 1000 medium was significantly greater than in RPMI 1640 medium, and the addition of 10% heat-inactivated fetal calf serum to CFBI 1000 medium usually did not produce a significant stimulatory effect on proliferation in contrast to the large effect observed in RPMI 1640 medium.

Effect of Aphidicolin on Thymidine Incorporation in CFBI 1000 Medium. Typically, addition of the DNA polymerase inhibitor aphidicolin (2.5 μ l/ml of medium; 2.5 mg/ml in dimethyl sulfoxide), which does not affect repair synthesis (9), either at the initiation of culture or 12 hr prior to the [³H]thymidine pulse, resulted in essentially background

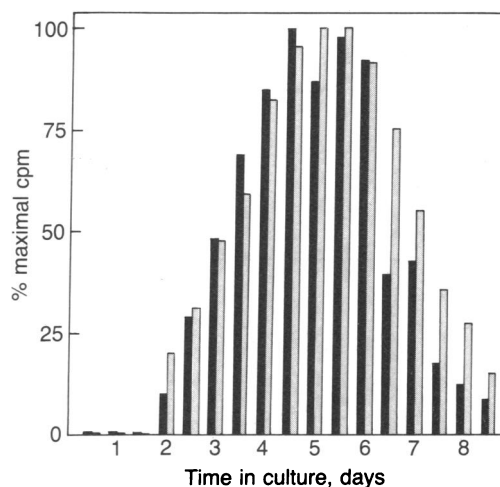


FIG. 1. Lymphocyte growth curves in CFBI 1000 medium. Lymphocytes were added to tubes or microwell plates at a concentration of 150,000 per ml in CFBI 1000 medium and incubated for the indicated periods of time. The cells were pulsed with [³H]thymidine 12 hr prior to harvesting. Solid bars indicate growth in tubes, and shaded bars indicate growth in plates. The cpm incorporated have been normalized to allow direct comparison.

incorporation of label into DNA [136 ± 42 cpm vs. $11,150 \pm 1061$ cpm (mean \pm SD) for medium alone]. Dimethyl sulfoxide utilized for addition of aphidicolin sometimes exerts a small effect when added at the initiation of the culture.

Response of Lymphocytes to Variation in Mitogen Concentration. Dose–response curves for PHA and Con A in CFBI 1000 medium have been determined for cells from two individuals (Fig. 3). The PHA used was not highly purified in order to ensure that the dose–response maximum covers a relatively broad range of concentrations. In contrast, the maximum for highly purified Con A is quite sharp, and higher concentrations are inhibitory. The concentration of Con A that elicited maximal response was normally 0.5 μ g of mitogen per ml of medium (Fig. 3). The maximum for PHA varied between 1 and 5 μ g of mitogen per ml of medium. Inhibition at higher concentrations was less pronounced than with Con A. No major differences in lectin response were noted between plates and tubes. These responses to mitogens are consistent with those reported in the literature for RPMI

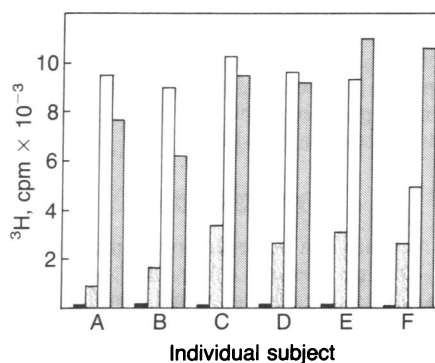


FIG. 2. Comparison of lymphocyte growth in CFBI 1000 and RPMI 1640 media in plates. Lymphocytes (30,000 per 0.2 ml) were inoculated into media, grown for 4 days, pulsed with [³H]thymidine, and allowed to incubate for 20 hr prior to harvesting. Solid bars indicate RPMI 1640 medium; stippled bars indicate RPMI 1640 medium with 10% fetal calf serum; open bars indicate CFBI 1000 medium; and hatched bars indicate CFBI 1000 medium with 10% fetal calf serum.

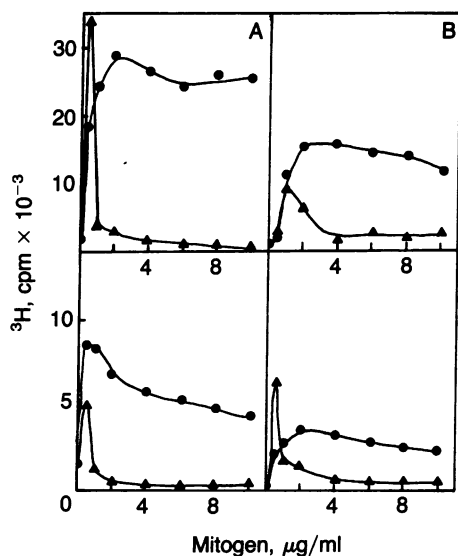


FIG. 3. Dose-response for lectins in CFBI 1000 medium. Lymphocytes were inoculated into CFBI 1000 medium at a concentration of 150,000 per ml at the indicated concentrations of the lectins, PHA (●), and Con A (▲). Cells were grown, pulsed with radiolabel, and harvested as described in the legend to Fig. 2. The upper panels are tube cultures, and the lower panels are microwell cultures. A and B are results from two different individuals.

1640 and other media used for growth of human peripheral blood lymphocytes (10, 11). For routine culture, a concentration of 2 μg of PHA per ml was utilized.

Variation in Maximal [^3H]Thymidine Incorporation Among Cells from Different Donors. Some individual variation was observed in the maximal amount of radiolabel incorporated into DNA over time (Figs. 3 and 4). However, in general, more variability was observed between cells from different individuals than in cells from the same person assayed at different times. The histograms in Fig. 4 demonstrate the variability between persons and between assays carried out at times as disparate as 2 years apart. Differences among individuals for a variety of parameters have been observed consistently in these studies: the maximal amount of radiolabel incorporation, the response to added serum, and the dose-response to mitogen and to serum are highly dependent on the individual. No correlation was observed between diminished radiolabel incorporation (e.g., Fig. 4, individual C) and stimulation by fetal calf serum. This sort of variability in the lymphocyte proliferation system has been widely reported (11–14), with differences in the amount of mitogen required for maximal stimulation, in the amount of radiolabel incorporated, and in the time for maximal radiolabel incorporation (15–18).

DISCUSSION

CFBI 1000 medium is a buffered solution containing simple compounds and no protein other than mitogen. Only 13 amino acids are required for maximal lymphocyte growth in most subjects. However, we have found that serine and/or glycine are not synthesized in optimal amounts for some individuals (unpublished results), and these two amino acids are accordingly included in the medium for routine applications. The absence of a requirement for added trace metals may derive either from the short-term culture conditions with insufficient multiplication to deplete cellular stores or from impurities present in the components used to formulate the medium. Serum is not utilized in the formulation, and no protein other than mitogen is added. The growth levels

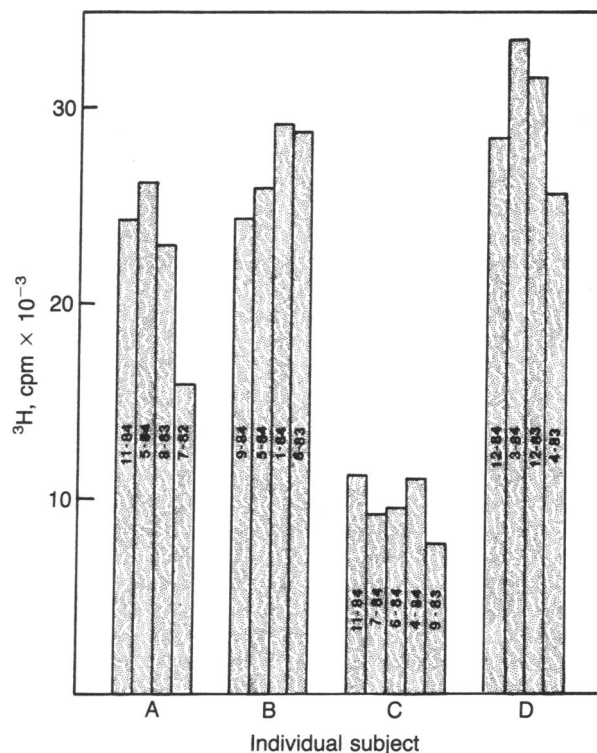


FIG. 4. Comparison of individual responses in CFBI 1000 medium over time. Lymphocytes were inoculated into tubes at a concentration of 150,000 per ml in CFBI 1000 medium and grown as described in the legend to Fig. 2. Culture at different times for the same individual resulted in the patterns indicated. The span of time covered by the different assays was 28 months for A, 15 months for B, 14 months for C, and 20 months for D.

achieved in this medium at low lymphocyte concentrations, as monitored by incorporation of [^3H]thymidine into DNA, exceed those obtained in RPMI 1640 medium, which contains 10% fetal calf serum. In the CFBI 1000 medium, the addition of fetal calf serum did not significantly alter the extent of radiolabel incorporation, in contrast to RPMI 1640 medium in which serum is essential. Preliminary evidence suggests that levels (and/or ratios) of amino acids are at least one source of significant inhibition in RPMI 1640 medium. The level of iron in CFBI 1000 medium may be another crucial factor.

Recently, several commercially available media for lymphocyte culture, including RPMI 1640 medium, Dulbecco's modified Eagle's medium, Ham's F 12 medium, and Iscove's modification of Dulbecco's medium, have been supplemented with proteins, including transferrin, insulin, albumin, lactoferrin, or mixtures of these proteins, and lipids to replace serum (11, 19–24). Growth of lymphocytes from most individuals is unaffected by addition of insulin or transferrin to the CFBI 1000 medium (data not shown). The absence of transferrin stimulation is in contrast to many other systems (25). Other stimulatory factors described in the literature (e.g., linoleic acid alone or conjugated with fatty acid-free albumin, ethanolamine, or α -mercaptoethanol) had minimal effect on lymphocyte growth response in CFBI 1000 medium (data not shown).

Response of a resting lymphocyte to mitogen includes blastogenesis and subsequent DNA synthesis associated with cellular proliferation. This DNA synthesis is monitored by incorporation of [^3H]thymidine into cellular DNA. Aphidicolin, an inhibitor of DNA polymerase, abolished [^3H]thymidine incorporation into lymphocytes in CFBI 1000 medium. Aphidicolin does not inhibit DNA repair (9) nor does it affect mitochondrial DNA synthesis (26). Thus, the incorporation of radiolabel observed in the CFBI 1000 medium

reflects *de novo* DNA synthesis and can therefore be considered a measure of cellular proliferation.

Individual growth responses differ markedly in this system. Roberts (12) noted that in medium containing serum there were significant variations in mitogen-induced proliferation for lymphocytes from different individuals and in cells from the same individual assayed at different times. In the CFBI 1000 medium, less variability in total response over time was observed for the same individual, although marked differences were found between individuals. Mendelsohn *et al.* (11) also have reported significant person-to-person variation in growth response. The basis for the variability is unknown, although the extent of monocyte presence in the lymphocyte fraction may be a potential source (13, 27). Experiments are necessary to determine the population size and types of cells proliferating as well as effects of adherent cell depletion on the lymphocyte response in CFBI 1000 medium.

The simplicity of CFBI 1000 medium allows variation in medium in order to study single limiting processes. For example, the omission of serine and glycine does not significantly decrease the growth response observed after 5 days in tube culture for most individuals, provided that the folic acid concentration is increased about 10-fold in the medium (data not shown). This modified medium provides a system to determine the effectiveness of serine and glycine biosynthesis and their interconversion in different individuals as well as the folic acid and pyridoxine coenzyme status of these biosynthetic pathways (2). Omission of riboflavin from the medium results in a significant decrease in lymphocyte response, which is quite variable for different individuals but markedly consistent for a single individual's lymphocytes cultured at different times over a period of several weeks. Thus, the medium components can be manipulated in a wide variety of ways to stress specific metabolic pathways to assess these processes in different individuals. A defined medium also has broad application in the study of the immune response mediated by the T-lymphocyte population. The effects of the multiple protein factors that modulate the immune response [e.g., neuropeptides (28) and gonadal steroids (29)] can be more easily assessed in a medium that is free of other protein components.

The availability of a medium that is easily formulated, is readily varied in composition, and is serum- and protein-free provides a useful tool for a broad range of scientific inquiry. The ease of manipulation facilitates both metabolic and immunologic studies, and the high growth response provides a base for investigating both stimulation and inhibition by other factors. The number of applications for such a system is quite large, and the results of using this medium should be of import in a variety of systems.

We acknowledge the contributions of the following people to the development of this medium: Beth Albright, Joyce Boghossian, Sherri Boykin, Lori Brotman, Martha Glenn, Charese Lande, Dea Larsen, Diane Maia, Ann McClain, Ann Mullin, Robert Read, Rosalva Santos, Gary Smith, Dan Wiginton, and Sarah Williams. This work was supported by the Clayton Foundation for Research.

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