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NUTRITIONAL REQUIREMENTS FOR GROWTH OF HUMAN LYMPHOCYTES

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INTRODUCTION

Mammalian cell culture has become an important avenue for the study of biochemical, nutritional, and developmental processes. The determination of specific growth requirements for cells in culture has been an area of intense investigation over the past several decades (see, for example, 3, 4, 44, 62,

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75). A distinction can be made between nutritional requirements for cells, other essentials for cell growth (e.g. oxygen, carbon dioxide, pH, temperature control), hormonal interactions (e.g. insulin), specific growth factors (e.g. nerve growth factor, epidermal growth factor), and mitogens. The human peripheral blood lymphocyte provides an excellent system for exploring biochemical and nutritional aspects of cell growth. This cell type is readily available, is in a resting state in terms of cell division, and can be stimulated to proliferate by exposure to a suitable stimulus (mitogens, antibodies, antigen, etc).

Lymphocytes have many metabolic pathways in common with other cells as well as a wide variety of cell surface receptors. Determination of cellular requirements for proliferation requires increased definition of the components of culture medium, both in type and amount (4, 36, 62, 75). The unknown composition and variability of commonly used serum supplements introduce a number of complexities into experimental design that can be avoided with a defined medium (3). In addition, many experiments cannot be executed without knowledge of all components in the medium. The development of a minimal medium that supports the proliferation of human peripheral blood lymphocytes provides a means not only to assess requirements for lymphocyte cell growth (66), but also to carry out individualized studies of nutrition and metabolism using lymphocytes (64, 65).

The purpose of this review is to survey the development of chemically defined media for growth of human lymphocytes and to indicate applications that are now possible with such media. It must be noted, however, that the requirements for activation and short-term growth of lymphocytes described herein may differ from those for long-term culture of cells over many generations.

MEDIA FOR GROWTH OF HUMAN LYMPHOCYTES

Serum-Containing Media

Fetal bovine serum has been used to supplement many different types of media to support growth of human peripheral blood lymphocytes in culture. The most commonly used medium for examination of human lymphocytes is RPMI 1640 (52), with varying concentrations of fetal bovine serum as one of the supplements. Serum contains the factors necessary for the growth of cells, but the presence of serum precludes many types of study that are of interest in this system (3).

Serum-Free Media

The problems associated with culture of cells in medium containing serum as a supplement have led to the development of media which support the growth of lymphocytes under serum-free conditions. To determine the effects of

polypeptide growth factors (which may occur in variable amounts in serum), to avoid the inhibitory properties of compounds in serum, and to eliminate influence from unknown components (even minor ones on a percentage basis) require serum-free media. The components essential for achieving a serum-free medium that supports a level of response comparable to (and frequently exceeding) the response in the presence of serum are transferrin, lipids, albumin, and in some cases growth factors (35, 36, 59, 68). Albumin was eliminated from such media with the addition of insulin, ethanolamine, and selenium as required supplements (47, 54). These components displayed additive growth-promoting effects when used in F12/DMEM (1:1) as basal medium with additional glutamine and antibiotic/fungicide supplementation (47). The effects of added lipids on proliferation in albumin-free media indicate that fatty acid sources may be of importance in the growth of cells, and the observed promotion of growth by albumin has been ascribed to bound fatty acids (36, 69). Even low levels of albumin may function in this capacity.

A major difference between serum-supplemented and serum-free cultures appears to be the relative rate of proliferation; variability in response of lymphocytes has also been noted (47). Mendelsohn et al (47) demonstrated that basal media supplemented with ethanolamine, transferrin, insulin, and selenium would not support long-term growth of interleukin-2-dependent human T cells or permanent lymphocyte cell lines. However, addition of albumin and a source of lipid was sufficient for growth of these latter cells. Casein has been used successfully to replace albumin, consistent with its role as a carrier of lipids (14). In some cases, the requirement for high levels of albumin and casein is eliminated by addition of catalase; this observation indicates that at least one essential function of serum and substitute proteins may be prevention of peroxide damage to cells (15, 16).

A defined basal medium for examination of murine T cells in vitro was derived by Gersten & Cohn (28) based on Iscove's modification of Dulbecco's medium (IMDM). Supplements of low levels of bovine serum albumin, transferrin, and insulin as well as mercaptoethanol, L-glutamine, and gentamicin further enhanced the preparation over the basic IMDM medium. The elimination of albumin and lipids did not significantly decrease the responses measured, in contrast to some observations on human lymphocytes (59, 68, 69). A general observation of growth characteristics in media without serum and with minimal protein supplements is the absence of nonspecific T-cell proliferation; the background level of T-cell division is significantly decreased, presumably by elimination of a broad range of growth factors associated with various supplements. In contrast, background stimulation and immunoglobulin secretion from human B cells may be increased in serum-free media (41, 71).

Another modification of IMDM includes addition of defined lipids, transferrin, bovine serum albumin, and α-thioglycerol (C-IMDM) (41). Compari-

son of growth of human peripheral blood lymphocytes in this medium with the standard RPMI 1640 supplemented with fetal bovine serum demonstrated greater sensitivity and response in the serum-free medium (41). RPMI 1640 medium supplemented with glutamine, gentamicin, magnesium, and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) will support proliferation of human lymphocytes, although higher cell concentrations are required to obtain a response comparable to serum-supplemented RPMI 1640 medium (55). Addition of lipoproteins and transferrin to RPMI 1640 supplemented with glutamine yielded mitogen-induced proliferation comparable to the same medium supplemented with serum (12).

Most of these studies examined growth in short-term cultures. Long-term culture in serum-free medium has been achieved utilizing casein, insulin, testosterone, transferrin, and linoleic acid as supplements to DME:Ham's F12 (1:1) (33, 53) to generate a medium that will also support short-term proliferation of human lymphocytes (14, 16). T lymphoblasts can be maintained in this medium for up to five weeks with supplements of 2-mercaptoethanol and 20% volume of this same medium conditioned by culturing human lymphocytes stimulated by phytohemagglutinin for 48 hours (14). Yen & Duigou (78) have used Ham's F12 medium supplemented with insulin, transferrin, albumin, and cholesterol and found it to support the growth of a human lymphocyte cell line. Ham's F12 medium contains a fatty acid (RPMI 1640 has none) and cholesterol; both factors may be essential for maintenance of the cell line as opposed to short-term culture.

Lymphokines

Lymphocytes require specific macromolecular factors termed lymphokines for growth, which, although not essential components of the basal medium, are released from cells into the medium. This biochemical cross-talk involving macrophage and various lymphocyte subpopulations is a requirement for cell division following stimulation (38). Interleukin-1 produced by macrophages apparently potentiates the T-lymphocyte response to mitogen (29) and serves as a mediator stimulating interleukin-2 production by T lymphocytes (19, 39, 67). Although a number of activities are ascribed to interleukin-2, which has been purified (27, 51, 76), its distinguishing feature is its ability to maintain growth of IL-2-dependent cell lines in vitro.

Recently, Herzberg & Smith demonstrated that serum proteins are required for early, optimal interleukin-2 production and interleukin-2 receptor expression on the cell surface (G_0-G_1 transition of T-cell activation), but not for later events in proliferation (33a). Supplementation of RPMI 1640 with additional glutamine, BSA, transferrin, and interleukin-2 yielded maximal T-cell proliferative response, although 48 hours later than for corresponding serum-containing cultures (33a). Serum proteins also function passively in prevent-

ing adsorptive loss of interleukin-2 produced by the cells; this role may be replaced by a variety of proteins or increased cell concentration (33a). These processes have not yet been explored in detail in defined media, and the influence of serum factors in the cascade of events leading to proliferation has not been delineated clearly.

Chemically Defined Serum- and Protein-Free Minimal Media

More than two decades ago, Ham developed the first chemically defined, synthetic medium that supported single-cell plating and serial propagation of two Chinese hamster cell lines; he then adapted this medium with slight modification for growth of a strain of mouse L cells (33). Subsequently, a related medium that allowed maximum growth of WI-38 diploid human fibroblasts when supplemented with 3% serum was used to demonstrate a selenium requirement of human cells (46). This and another closely related medium, MCDB 104, supported a low and variable growth of mitogen-activated lymphocytes in the absence of serum (65) and were used as the starting point to develop a serum- and protein-free medium. The amount of each constituent of the medium required for optimal response was determined. Components lacking stimulatory effects on human lymphocyte proliferation were omitted, and essential components were added in amounts just sufficient to generate an optimal response. Each adjustment of an individual constituent required a reevaluation of the dose-response curves of the remaining components. Only those substances required for the growth of lymphocytes from a substantial proportion of the subjects tested were included in the minimal medium. The use of the lowest possible concentration of each medium component was adopted for two reasons: (*a*) to minimize imbalances that might exert inhibitory effects, and (*b*) to diminish the level of any trace contaminants of medium ingredients. This approach yielded the chemically defined serum- and protein-free medium for mitogen-activated transformation of human peripheral lymphocytes, CFBI (Clayton Foundation Biochemical Institute) 1000 (45, 66).

In the preparation and utilization of a minimal medium, one must consider the sensitivity of cellular proliferative response to the formulation of the medium (e.g. order of addition of components, mode of pH adjustment) and also the water quality. It is essential that the water be free of low levels of organic compounds, chlorine, and other contaminants (73, 74). In addition, cells cultured in a minimal medium can be sensitive to compounds in the environment (e.g. carried in the CO_2 or air).

ESSENTIAL COMPONENTS The medium composition essential for mitogen activation and initial cell division of peripheral blood lymphocytes includes an appropriately buffered solution of inorganic salts, glucose or a related carbo-

hydrate source, amino acids, vitamins, and a few stimulatory components not synthesized by the cells in adequate amounts (45, 65, 66). Each of these classes is discussed separately.

Inorganic components Calcium, ferrous or ferric, potassium, magnesium, and phosphate ions, in addition to a physiological level of saline, are required for the transformation response of lymphocytes in the CFBI medium. Early attempts to obtain chemically defined media for animal cell growth utilized Earle's balanced salt solution (21, 22), which contains all of these components. Trace elements, which are undoubtedly required for metabolism, are either carried over in the cells or are present as contaminants in the commercial media components.

In CFBI 1000 medium, transferrin can replace ferrous (or ferric) salts complexed with EDTA. The level of iron required in the medium is high (10^{-5} M) relative to that of transferrin required to replace iron salts (10 μg/ml), presumably because of the efficiency with which transferrin delivers iron to the cell interior (58). Recent results from other laboratories demonstrate the role of transferrin to be exclusively in providing iron to proliferating cells (6, 7, 43). Many cell culture media, such as RPMI 1640 (52), which is used frequently for lymphocytes, do not contain iron salts; however, supplements of serum or purified transferrin provide the essential iron. The ability to eliminate transferrin as a medium component increases the definition of the medium, because contaminating compounds that may bind to the protein and may not be identified easily are eliminated. Trace metal contaminants, however, may be present in ferrous sulfate and EDTA; these unknown components may either retard growth or serve as required, but undefined, medium components.

Abboud et al obtained maximal DNA synthesis with 270-μM calcium and 100-μM magnesium (1), whereas other workers have found slightly higher levels are necessary for maximal thymidine incorporation in culture (10). Individual variations in these requirements exist, but for the lymphocytes of most individuals the CFBI 1000 concentration of 1 mM of these ions is adequate for maximum growth response.

Experiments with other media to determine requirements for trace metals have relied upon their removal from culture media and sera (48). Chelators coupled with an insoluble matrix were utilized to bind and remove the ions to avoid direct exposure of cells to the chelating species. For example, removal of zinc from medium and serum by chelation reduced lymphocyte growth response; this response was restored by addition of zinc to the culture medium (48). Similar results were obtained with copper and magnesium depletion (25). It is noteworthy that murine T-cell proliferation is much more sensitive to depletion of zinc, copper, and magnesium than is B-cell proliferation (25).

Trace metals are not required for growth in CFBI 1000, presumably because cellular reserves are adequate for short-term culture or because they are present as contaminants in other medium components, as previously discussed.

Carbohydrates A source of carbohydrates is essential for growth of lymphocytes, and virtually all cell culture media include glucose at concentrations comparable to blood levels. Some carbohydrates, e.g. mannose, can replace glucose, and others that are capable of being converted to some extent to glucose, e.g. galactose, can partially replace glucose. The effectiveness of these sugars is presumably related to the ability of the cell to convert these compounds into glucose.

Amino acids Early attempts to define the amino acid requirements for culturing animal cells used media containing the amino acids essential for intact animal growth; however, in culture of chick heart fibroblasts glutamine was identified as an additional important component (24). In a glutamine-containing medium supplemented with dialyzed horse serum, twelve amino acids were found to be essential for culturing mouse fibroblasts (L-strain) (21). Although media containing these thirteen amino acids were found to be sufficient for a number of human cell strains serially propagated in heavily seeded monolayer cultures, other "nonessential" amino acids, notably serine, were necessary for growth from small inocula (42).

In CFBI 1000 medium, the same thirteen amino acids are essential, with minimal response in the absence of any one of these amino acids. However, in addition to these amino acids, serine and/or glycine are not synthesized in adequate amounts for maximal cell growth by the lymphocytes of a significant number of individuals; these two amino acids are therefore included in the basal medium. Among the twenty amino acids common to protein biosynthesis, alanine, proline, aspartic acid, asparagine, and glutamic acid are not essential to generate an optimal response during the initial growth of mitogen-activated lymphocytes from most human subjects and are not components of the CBFI 1000 medium (66). This omission allows simple modifications of the basic medium to screen for the adequacy of synthesis of these amino acids in lymphocytes.

Many individuals synthesize sufficient glycine and serine for optimal lymphocyte growth responses if the 5-formyltetrahydrofolic acid content of the CFBI 1000 is increased about 10-fold (see below). Vitamin B_6 in the medium is required for this maximal response. These observations have been used to produce modified CFBI 1000 media in which the lymphocytes' ability to synthesize serine and/or glycine can be assessed. In addition, the availability of 5-formyltetrahydrofolic acid and vitamin B_6 for the synthesis and in-

terconversion of these two amino acids can be determined by using such media. For lymphocytes from most subjects, glycine alone is highly effective in replacing serine. Exceptions appear to be related to a vitamin B_{12} deficit. On the other hand, 5-formyltetrahydrofolic acid supplements are essential for effective utilization of serine to replace glycine.

In Eagle's minimal essential medium (MEM) supplemented with 20% dialyzed human serum, antibiotics, glutamine, calcium chloride, Hepes buffer, and other nonessential amino acids except serine and glycine, either serine or glycine was required for nearly maximal growth, but serine was somewhat more effective than glycine (20). Also, increased cell density enhanced growth in the absence of both serine and glycine. Extensive studies on RPMI 1640 supplemented with 10% dialyzed fetal calf serum have demonstrated that in this medium sufficient serine is not synthesized for optimal lectin transformation of lymphocytes (61). Thus, the ability of lymphocytes to synthesize glycine and/or serine is very sensitive to medium composition in addition to individual variations.

Methionine dependency of transformed human B lymphocytes and murine lymphocytes in culture has been reported (31, 37); however, the lymphocytes of many human subjects can respond in CFBI 1000 medium to homocysteine in lieu of methionine if adequate vitamin B_{12} is available (70). Methionine synthetase activity examined in lymphocytes under various conditions appears responsive to the addition of vitamin B_{12} to the system (32).

Although there are reports of asparagine requirements for human T-lymphocyte cell lines (57), proliferation in the CFBI minimal medium does not require asparagine for most individuals. However, a growth response to asparagine can be useful in indicating inadequacy of asparagine synthesis. The cellular requirement for L-glutamine appears to be absolute; these experiments were conducted only with B cells in RMPI 1640 medium with 10% dialyzed and heat-inactivated fetal calf serum (11). [^{3}H]-Thymidine incorporation was restored with as little as 0.08-mM L-glutamine, although immunoglobulin synthesis increased with higher levels of glutamine in the medium (11). L-Glutamine is present in CFBI 1000 minimal medium at 0.4 mM, a level sufficient for maximal [^{3}H]thymidine incorporation in lymphocytes from most individuals upon stimulation with phytohemagglutinin.

Vitamins The short-term mitogen activation and initial growth of activated lymphocytes over a period of five days result in about three cell divisions. Consequently, if significant reserves of an essential catalytic factor are present in the cells, the medium may be free of this component while still supporting optimal growth. However, the size of the inoculum has an effect: small inocula show a greater relative effect upon the omission of a catalytic factor from the medium.

The eight B vitamins known to be required for animal growth along with lipoic acid are constituents of the chemically defined medium developed for the growth of human fibroblasts (46). Lipoic acid does not usually exert any effect on short-term lymphocyte growth and is accordingly omitted from the minimal medium, CFBI 1000. The essential nature of the eight B vitamins can be demonstrated in various ways. Omission of a vitamin, e.g. pantothenic acid, biotin, or riboflavin, from the medium usually causes a substantial decrease in the growth response when a small inoculum of lymphocytes is used. The advantages of a minimal medium are well illustrated by the direct dose-response effect of biotin on the growth of lymphocytes in CFBI 1000 (see 45), in contrast to the difficulty experienced in demonstrating a biotin requirement for human cells in complex medium containing unknown components (13).

Responses to folic and 5-formyltetrahydrofolic acid are best demonstrated in a glycine-free medium containing serine in which the conversion of serine to glycine is the growth-limiting step. The ability of homocysteine to replace methionine is dependent upon vitamin B_{12} supplements (32, 61). Vitamin B_6 stimulation of lymphocyte growth can be effectively demonstrated in the absence of both serine and glycine. The decreased responsiveness of lymphocytes to vitamin B_1 is enhanced by substituting a ribosyl derivative for the glucose; this is because of the consequent dependence on transketolase for conversion of the sugar to required glucose. Metabolic antagonists as inhibitors of vitamin utilization are useful not only in demonstrating the requirement for such catalytic factors but also in the development of assays for the adequacies of cellular levels of such factors (65, 70).

The formation of formate, and presumably kynurenine, from tryptophan occurs in lymphocytes (61); kynurenine is a precursor of the nicotinamide moiety of the coenzyme (NAD^+). The NAD^+ concentration in human lymphocytes increases proportionately with cell size following stimulation (77), and it is possible that not only the coenzyme (NAD^+) but also the vitamin is synthesized. Supplements of nicotinamide or nicotinic acid do not affect the growth of peripheral lymphocytes from most individuals, but diminished growth of lymphocytes of some individuals upon omission of this vitamin does occur.

Lymphocytes have been used as a marker of past nutritional state for individuals with deficiency of folate and vitamin B_{12}. Using deoxyuridine suppression of radioactive thymidine incorporation into DNA, Das & Herbert (17) demonstrated that patients with megaloblastic anemia due to deficiency of folate or vitamin B_{12} did not exhibit suppression of thymidine incorporation in the presence of deoxyuridine. The deficiency state in lymphocytes persists for some time after anemia has been eliminated in treated patients. In vitro, these abnormal patterns were corrected by the addition of folate, methylfo-

late, and vitamin B_{12} to the medium. These studies demonstrated that vitamin deficiencies can be corrected in vitro and that the lymphocytes reflect the vitamin status of the patient at the time the lymphocytes were generated.

The uptake of vitamin B_{12} into peripheral human lymphocytes was determined as a function of cell cycle (30). Minimal uptake was found for resting lymphocytes, but increases were observed upon stimulation with mitogen, apparently as a result of increased levels of receptor activity for transcobalamin-II-cobalamin. The ability to transport vitamin B_{12} apparently occurs over a relatively narrow time window correlating with the period of most active DNA synthesis. All these studies employed RPMI 1640 with fetal calf serum as a supplement.

The fat-soluble vitamins have not been routinely included in media for the growth of human lymphocytes, in part because of solubility problems and in part because there is no evidence that they are required for cellular proliferation. In fact, inhibition of human B-cell response to activation by suboptimal levels of pokeweed-mitogen has been demonstrated using 1α,25-dihydroxyvitamin D_3 in serum-free culture conditions (63). The effect was not observed for 24-R,25-dihydroxyvitamin D_3 or 25-hydroxyvitamin D_3. The hormonal form of vitamin D_3 also inhibited lymphocyte proliferation in response to phytohemagglutinin, apparently by suppressing interleukin-2 production (72). This effect was observed in RPMI 1640 with fetal calf serum at extremely low concentrations of 1α,25-dihydroxyvitamin D_3 ($\sim 10^{-11}$ M) and was partially reversed by adding purified interleukin-2 (60). The inhibition is time-dependent in that addition of this hormone 12–18 hours after mitogen exposure reduces its suppressive effects; this observation may indicate an indirect influence, since calcitrol receptors do not appear on T lymphocytes until 24 hours after stimulation. The suppressive effect of 1α,25-dihydroxyvitamin D_3 has been shown to be T-helper specific (40).

Other medium components Omission of pyruvate, inositol, or adenine from the minimal medium usually diminishes growth response by 30–60%, although there is considerable individual variation (66). For specific individuals, the effect of omission of pyruvate can range from negligible to almost total suppression, presumably related to the effectiveness of the lymphocytes in carrying out glycolysis. Inositol and purines generally are not synthesized by lymphocytes in sufficient concentrations for optimal growth. Precursors of purines, e.g. 5-amino-4-imidazolecarboxamide, can enhance the response in the absence of adenine. For most individuals, a substantial decrease (70–90%) in response in the absence of choline occurs, but certain precursors can replace choline. Synthesis of choline appears to be greatly restricted in the lymphocyte.

ACTIVATION AND STIMULATION OF GROWTH

Lectins

The primary method for activating peripheral human lymphocytes in culture is mitogen stimulation; phytohemagglutinin is the most commonly used mitogen. One study indicated that this lectin may activate T-lymphocyte transformation by binding to the T_i portion of the antigen receptor (5); however, other studies have indicated that phytohemagglutinin exerts its action via the T11 receptor (sheep red blood cell receptor), although concanavalin A does not (56). Concanavalin A is also capable of stimulating T-lymphocyte response, while lipopolysaccharide elicits proliferation of B lymphocytes (34). Pokeweed-mitogen activates both T and B lymphocytes (34). Phytohemagglutinin isolectins L4 and E4 have been compared in their transformation capacity (23). In the presence of serum, L4 was 30–60 times more potent as a mitogen than E4, whereas under serum-free conditions, the differential decreased to less than 10-fold (23). These differences illustrate the potential interactive effects of serum on various medium components, including lectins. All of these mitogens have been successfully used in the CFBI 1000 defined medium with approximately comparable responses (45).

Antibodies to Surface Antigens and Mixed Lymphocyte Culture

Lymphocytes can be activated in short-term culture by exposing the lymphocytes from one donor to irradiated cells from another donor. The latter cell population provides the antigenic signal necessary to induce cellular events required for subsequent cell division. In some cases, antibodies specific for various receptor molecules on the surface of the lymphocytes activate the cells. Antibodies to the T_3-T_i antigen receptor complex or cross-linking of the T3 component of the antigen/MHC receptor with T4 antigen increase cellular proliferation (2, 49). Similarly, T-cell activation can occur via binding of CD2 (T, gp50), antibodies (8) or anti-T11 antibodies (26, 50). Most, if not all, of the experiments that have explored activation by specific cell surface antigen interaction with antibodies have utilized serum-containing media. Almost no exploration of the requirements for cell proliferation in minimal, defined media has been reported. The mechanisms by which serum influences the events or signals required for cellular activation is not yet known (33a).

Other Modes of Activation

The signal for proliferation can be provided at least partially by a combination of phorbol esters, which activate protein kinase C, and interleukin-1, which is

a product of the monocytes. The physical proximity of monocytes, however, may be essential for T-cell activation (18). Experiments that demonstrate the effects of phorbol esters in combination with ionophores and other lymphokines have all been executed in serum-containing medium. Again, whether or not this supplement influences the process of activation is unknown.

Proliferation can also be initiated in murine lymphocytes by the calcium ionophore A23187, if thiols are also present in the culture medium (9). Serum supplementation increases the extent of proliferation. It is apparent that multiple and diverse signals can elicit the proliferative response, and medium composition, particularly the presence of serum and proteins, can influence the extent of cell division.

APPLICATIONS FOR MINIMAL MEDIA

The development of a minimal medium (CFBI 1000) that will support the growth of human lymphocytes in the absence of serum and proteins makes it possible to define the requirements for this cell type in culture (66). Beginning with this formulation, it is then possible to vary the composition of the medium and test the effect on growth of lymphocytes from different individuals. This nutritional and metabolic evaluation is feasible because unknown components are eliminated to the extent possible with maximal definition of the medium.

With a defined minimal medium, the nutritional and metabolic growth studies so effectively utilized in studying microbial systems can now be adapted to analogous studies in human lymphocytes. For example, the extent of the decrease in response of lymphocytes to the omission of a catalytic factor such as pantothenic acid, biotin, or riboflavin rests not only on the quantity of cellular reserves but also on the ability of the cell to utilize these reserves. Consequently, broad variation in growth diminution exists in different individuals when a single required catalytic factor is omitted. The magnitude of this response is influenced by the adequacy of intake, deficits in transport not only into the circulatory system but also into the cells, inadequacies in the utilization of cellular reserves, and the intrinsic requirement for the factor (which may be increased by various causes, e.g. decreased affinity for an essential enzyme).

The number of subjects with substantial decreases in lymphocyte response in the absence of riboflavin in the medium correlated with decreased riboflavin consumption as measured by riboflavin excretion (65). Also, a good correlation was demonstrated between the vitamin B_6 status (determined with erythrocyte glutamate-oxalacetate transaminase) and diminished lymphocyte

growth consequent to omission of vitamin B_6 from a minimal medium not containing glycine (65). Complete correlation would not be anticipated, since growth can be influenced by altered coenzyme affinity for a number of different enzyme systems.

Dose-response data for required factors that have no significant accumulation in the cells can indicate abnormal requirements or metabolism. It is also possible to determine cellular sensitivities to inhibitory effects, e.g. amino acid imbalances. Individuals whose lymphocytes do not synthesize adequate amounts of particular metabolites can be detected easily by screening for growth stimulation in response to these compounds (an observation unusual with the lymphocytes of most subjects). Similarly, these procedures can detect deficits of trace elements or catalytic factors that, because of adequate cellular reserves, are not normally required for the lymphocytes of most individuals for short-term culture. Through the use of specific metabolic antagonists, essentially all of the metabolic processes can be examined. Thus, metabolic and nutritional assessments involving hundreds of media variations are now possible using this simple-to-modify, basal medium as the basis for identifying limiting nutritional and metabolic substances. The clinical effects of dietary supplementation of individual subjects with limiting factors identified by this type of assay may be correlated ultimately with the effectiveness of the lymphocyte culture to predict patient responsiveness.

Hormonal effects can also be determined in the CFBI 1000 minimal medium. For example, insulin decreases the amount of glucose required for growth of lymphocytes (65). However, for a minority of individuals insulin enhances growth, even at optimal glucose levels. Many of these subjects exhibit abnormal glucose tolerance tests. Growth of lymphocytes in minimal media also affords an excellent test system for sensitivity to toxicities of drugs and drug metabolites.

In addition to the applications for defined growth media in nutritional and metabolic assessment, such media are useful in isolation of growth factors produced by cells and secreted into the external environment. Attempts to separate such factors from serum- and protein-containing media require complex procedures to remove contaminating proteins. In contrast, separation from other secreted proteins is the only requirement in a minimal medium. In similar fashion, assessment of the effects of macromolecular components on growth is simplified in a minimal medium. Interactions with other proteins or macromolecules in the medium are avoided, and direct determination of the effects of a factor can be made readily. Furthermore, although serum may promote cellular proliferation, it also may inhibit the process; use of media free of sera avoids these complications and in fact provides a system in which the effects of various serum components can be determined.

CONCLUSIONS

The nutritional requirements of human peripheral blood lymphocytes in culture have been identified by the formulation of a minimal medium that will support in vitro proliferation in response to mitogens (66). It must be noted, however, that the "minimal requirements" vary significantly among individuals. Thus, general definition of the growth requirements for these cells may eliminate a component required for lymphocyte growth in some individuals. These differences may be exploited in determining individual requirements for cell proliferation; this information may be used to deduce an individual's nutritional/metabolic status by extrapolation from the in vitro response. Preliminary clinical results indicate that supplementation based on such assessment may be beneficial across a wide range of disease states (65; W. Shive et al, unpublished results). The development of CFBI 1000 minimal medium also provides a system for evaluating lymphocyte activation in response to a variety of stimuli without complications arising from unknown or unnecessary medium components. This medium has not yet been significantly exploited in this area.

In summary, the nutritional and metabolic requirements of human peripheral lymphocytes in cell culture are indicated by the relative growth of cells in different formulations of minimal, defined media. The required components are amino acids, vitamins, various ions, pyruvate, inositol, adenine, choline, and glucose as an energy source, all in a buffered system. This constellation of compounds can be systematically varied to stress a particular metabolic system, to determine a requirement for a particular compound, or to assess toxicity. The simplicity of the system lends itself to use as a tool for nutritional and metabolic assessment of human individuals.

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