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Modulation of human lymphocyte proliferation by amino acids

J. C. CHUANG, C. L. YU & S. R. WANG *Section of Allergy, Immunology & Rheumatology, Department of Medicine, Veterans General Hospital, and Institute of Microbiology & Immunology, National Yang-Ming Medical College, Taipei, Taiwan, Republic of China*

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SUMMARY

The amino acids required for phytohaemagglutinin (PHA) induced lymphocyte proliferation were determined by the ^{3}H-thymidine incorporation in amino acid-deficient media. Results indicate that the PHA-stimulated lymphocytes require alanine and serine in addition to 13 other amino acids present in Eagle's minimal essential medium (arginine, cysteine, glutamine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine, and valine). The omission of any one of the 13 amino acids would stop almost completely the proliferation of PHA-stimulated lymphocytes. The omission of serine from RPMI 1640 medium caused a mean reduction of 64% of cell proliferation, while the addition of alanine to PRMI 1640 culture medium caused a mean increment of 52%. The lymphocyte proliferation appears to be modulated by amino acids in the culture medium, and for optimal growth of lymphocytes, all these 15 amino acids are essential.

Keywords lymphocyte proliferation amino acids phytohaemagglutinin

INTRODUCTION

Mitogen-stimulated lymphocyte activation is an *in vitro* technique commonly used to assess cellular immunity in patients with immunodeficiency, autoimmune disease, infection or cancer (Oppenheim *et al.*, 1975). A variety of complex biochemical events, including synthesis of protein, RNA, and finally DNA, occurs following incubation with mitogens (Ahern & Kay, 1975 Varesio & Holden, 1980). The increase in DNA synthesis that eventually results in cell division forms the basis for most clinically relevant assays for lymphocyte activation.

In order to better understand the biochemical basis of the cellular immune response, we have studied the effects of amino acids on phytohaemagglutinin (PHA) induced lymphocyte proliferation by means of DNA synthesis.

Waithe *et al.* (1975) reported that 14 amino acids were required for protein synthesis in lymphocytes. These amino acids include arginine (Arg), cysteine (Cys), glutamine (Gln), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), serine (Ser), threonine (Thr), tryptophan (Trp), tyrosine (Tyr) and valine (Val). As for DNA synthesis, no persuasive study has been done in which amino acids were required for lymphocyte proliferation.

In this report, the requirement for various amino acids that are prerequisite for DNA synthesis during lymphocyte activation was studied. Among them, alanine (Ala), which had been neglected in the past, was also studied.

Correspondence: Soo-Ray Wang, M.D., Ph.D., Section of Allergy, Immunology & Rheumatology, Department of Medicine, Veterans General Hospital, Taipei, Taiwan 11217, Republic of China.

MATERIALS AND METHODS

Lymphocyte preparation

Mononuclear cells (MNC) were separated from heparinized peripheral blood of healthy donors by Ficoll–Hypaque density gradient centrifugation as previously described (Wang *et al.*, 1984). The MNC were washed twice in HBSS and were suspended in the amino acid-free RPMI 1640 (GIBCO, Grand Island, NY). The cells thus obtained consisted of $92 \pm 2\%$ (s.d.) MNC, the rest being neutrophils. About 90% of MNC were small lymphocytes as determined by Wright staining, and the others were monocytes. Cell viability, as determined by trypan blue dye exclusion, was between 96% and 100%.

Culture media

Media containing all 20 amino acids (complete medium) and media lacking any one specific amino acid were prepared. Briefly, the sterile solutions of the appropriate L-amino acids were added to amino acid-free RPMI 1640 (obtained from GIBCO). The media thus prepared were further supplemented with 10% dialysed fetal bovine serum (FBS). The final concentration of each amino acid in the culture media was the same as that of RPMI 1640 (Moore, Gerner & Franklin, 1967).

FBS was inactivated at 56°C for 30 min, and was dialysed against nine changes of 40 volumes of 0·01 M phosphate-buffered saline (PBS) (pH 7·2) for 72 h at 4°C.

The PHA-stimulated lymphocytes cultured in complete medium supplemented with either dialysed or non-dialysed serum showed practically no difference in ^{3}H-thymidine (^{3}HT-dR) incorporation.

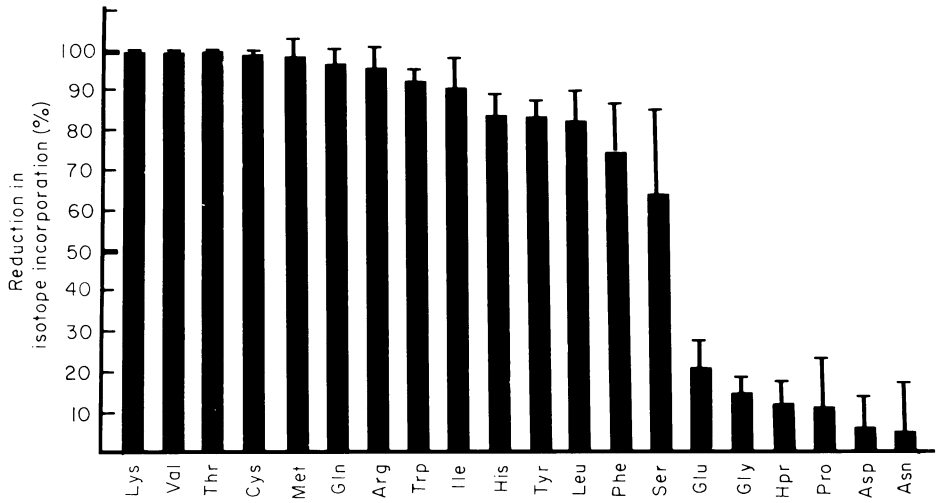

Fig. 1. The effect of amino acid deficiencies on ^{3}H-TdR incorporation ($n = 7$). The rate of ^{3}H-TdR incorporation was determined at 72 h of incubation with PHA. Each vertical bar represents the mean reductions of isotope incorporation which were expressed as percent fraction of incorporation in complete medium ± 1 s.d. (see Materials and Methods). The mean ct/min ± s.d. of cell cultures in complete culture media was 97 909 ± 25 344.

Lymphocyte cultures

Lymphocytes (5×10^5/ml) were cultured in 96-well flat-bottomed microtitre plates (Costar, Cambridge, MA). There was 0·2 ml of culture medium in each well containing 10% (v/v) FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 1 μg/ml PHA (Burroughs Wellcome, Dartford, UK). All cultures were set up in quadruplicate and incubated in a 5% CO_2 humidified incubator for 72 h. During the final 4 h of the culture period, these cells were pulsed with 0·5 μCi/well of ^{3}H-TdR (specific activity 6·7 Ci/mmol, New England Nuclear, Boston, MA). Cultures were harvested onto glass/fibre filters with an automatic cell harvester (model M24, Brandel). Isotope incorporation was measured by a standard toluene-based counting technique (Wang & Zweiman, 1978). The results were expressed as the arithmetic means of ct/min for quadruplicate cultures.

Expression of amino acid dependency

Amino acid dependency was calculated according to the following formula:

$$\text{Dependency} = \frac{\text{Complete} - \text{Lacking ct/min}}{\text{Complete ct/min}} \times 100\%$$

where complete ct/min represents ct/min of the culture in complete RPMI 1640 medium; and lacking ct/min represents ct/min of the culture in medium lacking the one amino acid under study.

The degree of amino acid dependency for lymphocyte proliferation was measured by comparing cell proliferations both in the complete culture medium and in the medium lacking the amino acid of interest. The amino acid was grouped as 'essential' when its depletion caused more than 80% reduction of cell proliferation; as 'partially essential' when the reduction was between 20 and 80%; and as 'non-essential' when the reduction was less than 20%.

RESULTS

The effect of amino acid deficiency on lymphocyte proliferation

In order to determine the amino acid dependency of lymphocyte proliferation, cells were stimulated with PHA in media lacking one amino acid. The effects of the depletion of each of 20 amino acids on the degree of ^{3}H-TdR incorporation in cell cultures were determined in seven experiments using lymphocytes from seven healthy donors. The results are summarized in Fig. 1, expressed as percentage of dependency in order to compare the individual experiments.

Twelve amino acids grouped as essential (dependency ≥ 80%) included Lys, Val, Thr, Cys, Met, Gln, Arg, Trp, Ile, His, Tyr and Leu. Ser and Phe were grouped as partially essential (dependency 20–80%). Six amino acids grouped as non-essential (dependence < 20%) include asparagine (Asn), aspartic acid (Asp), glutamic acid (Glu), glycine (Gly), hydroxyproline (Hyp) and proline (Pro). Ala was not included in the commercial RPMI 1640. Its addition in the culture medium caused a 44–62% increment of lymphocyte proliferation (Fig. 2). Therefore, it appears that Ala may be grouped as partially essential for lymphocyte proliferation.

DISCUSSION

The present study points to the complete dependency of human lymphocyte proliferation on 12 amino acids. Three amino acids are partially essential and six are non-essential.

Numerous observations attest to the importance of adequate protein nutrition for maintaining immune competence (Drenick & Alvarez, 1971; Chandra, 1972; Bang *et al.*, 1975); however, few studies have attempted to determine whether any single amino acid has a significant role in immune competence. Waithe *et al.* (1975) demonstrated the amino acid requirement in protein synthesis of PHA-stimulated lymphocytes. The pattern of amino acid dependency in protein synthesis is very similar to that in DNA synthesis, as shown in present study.

Chevalier & Aschkenasy (1977) reported that isolated deficits or excesses of a single essential amino acid, or an imbalance among essential amino acids, does seem to reflect itself in functional changes in humoral immunity. There is only one study concerning the effect of such amino acid imbalance on cell-mediated immunity (Jose & Good, 1973). The possible

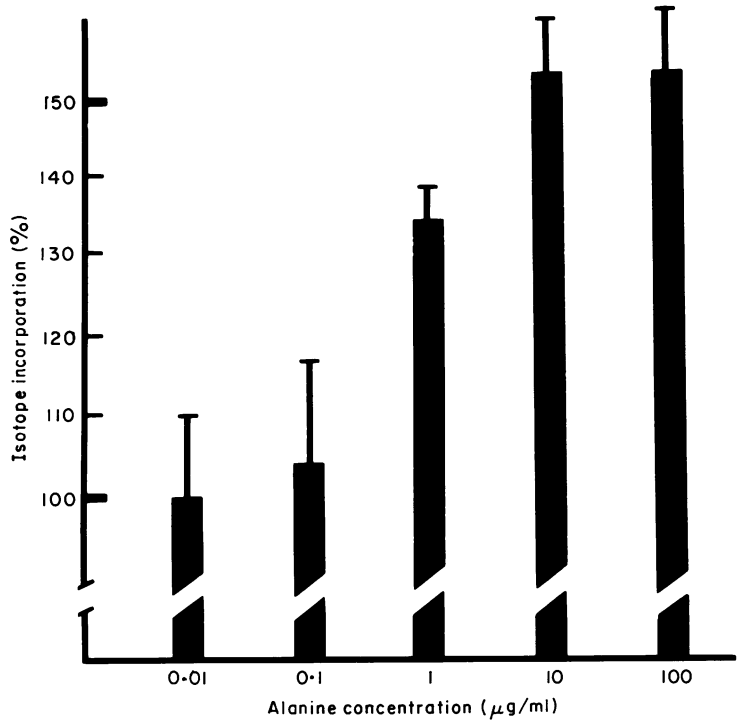

Fig. 2. The effect of L-alanine on the ^{3}H-TdR incorporation in RPMI 1640 cell cultures ($n = 4$). The incorporation was determined at 72 h of incubation with 1 μg/ml of PHA. The ordinate represents the mean (±1 s.d.) isotope incorporation. The incorporation by cells cultured in complete medium was taken as 100%.

effect of any single non-essential amino acid on immune function has not been well elucidated (Beisel, 1982).

In an animal model, Jose & Good (1973) reported that the moderate reduction in Phe-Tyr, Val, Thr, Met, Cys, and Trp in the diet of mice produced severe depression of humoral immune responses in terms of haemagglutinating and blocking antibodies, although their cytotoxic cell-mediated immunity was not impaired. The reduction of Lys, Arg or His in the diet caused only a slight depression of the humoral immune response.

In the human model, although eight amino acids are known to suffice for nitrogen balance in feeding experiments (Rose *et al.*, 1955), several cultured human cells and cell lines have been found to require 13 amino acids for survival and growth (Eagle 1955a, 1955b; Eagle *et al.*, 1956; Eagle, 1959). These 13 amino acids include five non-essential amino acids (Arg, Cys, Gln, His, and Tyr) in addition to eight essential amino acids (Ile, Leu, Lys, Met, The, Thr, Trp, and Val) for humans.

It was of interest to find that more amino acids were required for *in vitro* cell proliferation (15 amino acids) than for *in vivo* body growth (eight essential amino acids) for humans. It is possible that the lymphocytes under our *in vitro* study are unable or only able to synthesize very slowly those seven other amino acids which were not essential for humans *in vivo*.

Six amino acids (Asp, Asn, Glu, Gly, Hyp, and Pro) were not essential for lymphocytes. In their absence, the lymphocytes continued to proliferate at a nearly normal rate for 3 days. It was unlikely that the 10% of dialysed serum protein in the medium could supply these six amino acids, it would be reasonable to surmise that it would supply at least some of the other 15 amino acids. It was further noted that these six amino acids were also non-essential for humans *in vivo*.

Some amino acids, such as Ser, Asn, and Gly, are synthesized by mammalian cella. However, they are required by lymphocytes at low cell populations, i.e. less than 1×10^5/ml (Eagle & Piez, 1962). This population-dependent effect would not be expected to occur in our cultures which contained 5×10^5/ml of lymphocytes.

It is clear that only eight amino acids are essential for humans *in vivo* . However, it is not clear whether other amino acids are essential for individual organs. Our study has proven 15 amino acids to be essential for lymphocyte growth, an observation helpful in understanding the effect of amino acids in local immune responses, for example, the immune response in the liver. The liver contains an abundant quantity of arginase which is capable of depleting Arg., resulting in a state of local immune suppression (Brusdeilins *et al.*, 1983), but this deserves a further study.

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