CONCISE REPORT

Phytohemagglutinin-Stimulated Normal Human Peripheral Blood Lymphocytes in Folate-Depleted Medium: An In Vitro Model for Megaloblastic Hemopoiesis

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Normal human peripheral blood lymphocytes cultured with phytohemagglutinin in folate-free RPMI 1640 medium supplemented with normal human serum exhibit megaloblastic maturation. These changes are accompanied by a decrease in intracellular folate content, and when compared to folate-replete cells, revealed increased 3H-thymidine incorporation into DNA together with a decreased inhibitory effect of deoxyuridine on 3H-thymidine incorporation. This in vitro system of cellular folate deficiency is a convenient model for analysis of biochemical events accompanying megaloblastic maturation.

Much of the evidence for altered de novo pyrimidine biosynthesis in human cobalamin or folic acid deficiencies is centered around the demonstration of deficient proliferating cells of an abnormal deoxyuridine (UdR) suppression test. This procedure, which evolved from the observation that exogenous UdR inhibits or suppresses the uptake of radiolabeled thymidine into cobalamin-deficient marrow cells to a lesser degree than with normal cells, is generally regarded to discriminate between cellular cobalamin and folate deficiency.

More controversial are those questions pertaining to its biochemical basis or the nature of molecular mechanism(s) responsible for the expression of megaloblastic hemopoiesis. Progress in this area is dependent on the availability of deficient human cells and is clearly restricted by the lack of a suitable in vitro model. In this article we report the development of a reproducible in vitro model for cellular folate deficiency employing normal human peripheral blood lymphocytes stimulated, in folate-depleted culture medium, into mitosis by phytohemagglutinin (PHA).

MATERIALS AND METHODS

Preparation of Peripheral Blood Mononuclears and Culture Conditions

Normal human peripheral blood mononuclears were obtained by density centrifugation of whole blood on Ficoll-Hypaque and washed twice with folate-free RPMI 1640 medium (Flow Laboratories). Lymphocytes were cultured in microplates (Falcon 3040) containing 1.25 x 10^6 cells in a final volume of 0.25 ml. Unless otherwise stated, cell cultures contained 20% pooled normal human serum and folate-free RPMI 1640 medium containing glutamine (2 mM), HEPES (Calbiochem) buffer pH 7.4 (20 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and PHA-P (Wellcome) at 0.8 isg/ml. The plates were incubated in a humidified atmosphere commencing of incubation or together with UdR (0.1 mM) 5 hr prior to harvesting.

Measurement of Deoxynucleoside Incorporation in DNA

After 68 hr in culture, 0.5 μCi of either 6-3H-thymidine (5 Ci/m mole) or 6-3H-deoxyuridine (22 Ci/m mole, Radiochemical Centre, Amersham) were added to each well. The cells were harvested at 72 hr using an automated sample harvester (Skatron) and radioactivity in trichloroacetic acid precipitated material assayed in a liquid scintillation counter.

Cellular Morphology and Folate Content

Lymphocytes were cultured in a 1-ml volume in 12 x 75 mm plastic tubes (Falcon 2058), under conditions identical to those described above. For morphological studies, cells were harvested after 5 days, washed 3 times with isotonic saline, and suspended in pooled human serum. Prepared slides were stained by May-Grünwald-Giemsa. To determine cellular folate content, approximately 5 x 10^6 cells were harvested either prior to stimulation or at 72 hr, washed 4 times with ice-cold isotonic saline, pooled, and suspended in 1% ascorbic acid. After freeze-thawing in liquid nitrogen, the specimens were centrifuged at 4°C for 10 min at 5000 g. Folate levels were determined by a commercially available radioactive binding kit (Diagnostic Products).

RESULTS

The folate content of pooled human-serum-supplemented RPMI 1640 folate-free medium was 1 ng/ml; of unstimulated lymphocytes 320 ± 60 pg/10^6 cells (mean ± 1 SD), and that of cells after 72 hr in culture following exposure to PHA in either folate-depleted or folic-acid-supplemented (1 μg/ml) medium was 70 ± 40 and 4600 ± 400 pg/10^6 cells, respectively.

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The morphology of PHA-transformed lymphocytes in folate-depleted or supplemented cultures is illustrated in Fig. 1. The cells in folate-depleted cultures tended to be larger, with the voluminous nuclei exhibiting an open, finely stippled, and lacy chromatin pattern with usually more than two prominent nucleoli; the cytoplasm of these megalymphoblasts showed prominent vacuolization.

The effect of preincubation with UdR on tritiated thymidine ($^3$H-TdR) incorporation into DNA at 72 hr in either folate-depleted cultures or those supplemented with 0.1–1.0 μg/ml of folic acid (PGA), formyl-tetrahydrofolate (THFA), or methyl-THFA prior to addition of PHA is shown in Fig. 2. In the absence of added folates, the incorporation of $^3$H-TdR into DNA in cultures preincubated with UdR was 70% ± 5% (mean ± 1 SE) that of control cultures not incubated with UdR. The addition, ab initio, of the previously outlined folates resulted in a progressive increase in the ability of UdR to inhibit subsequent $^3$H-TdR incorporation; with concentrations of folates greater than 0.4 μg/ml, inhibition approached 10% that of control cultures. At the lower concentrations, a significant difference existed between the corrective effect of PGA or formyl-THFA and that of methyl-THFA ($p < 0.005$). The reduced UdR suppressive effect in folate-depleted cultures was also corrected by the addition of folates together with UdR. However, to achieve comparable degrees of inhibition of $^3$H-TdR incorporation to that observed when folates were added ab initio, required concentrations an order of magnitude higher. Initial supplementation of cultures with the folates resulted in a progressive reduction in the incorporation of $^3$H-TdR into DNA at 72 hr, being, at the highest concentration tested, 71% ± 3%, (mean ± 1 SE, $p < 0.001$) that of nonsupplemented cultures. No significant difference in this effect was noted between the individual folates. In contrast, the incorporation of $^3$H-UdR into DNA was increased threefold in cultures supplemented ab initio with PGA.
at 0.1 μg/ml, with no significant increase observed at high concentrations.

**DISCUSSION**

The morphological characteristics exhibited by mitogen-stimulated normal peripheral blood lymphocytes in the folate-depleted culture conditions described here are remarkably similar to those of marrow cells or transformed peripheral blood lymphocytes of individuals with either cobalamin or folate deficiency. These in vitro induced abnormalities appear to be consequent to intracellular folate depletion. That this is the case is suggested, first by the reduction of intracellular folate content of cells in folate-restricted medium pari passu with mitogen-induced events, in contrast to the increase of folate content relative to nondividing cells in lymphocytes stimulated in the folate-supplemented medium. Second, the expression by these megaloblastic cells of ineffective UdR inhibition of 3H-TdR incorporation into DNA and its reversal by folates is a finding consistently demonstrated in dividing cells with in vivo acquired cobalamin or folate deficiency. The corrective difference between methyl-THFA and other folates at lower concentrations in reversing the effect of UdR is not readily explicable, as the vitamin B12 concentration of the culture medium is not limiting. This difference has also been documented for PHA-stimulated lymphocytes with in vivo acquired folate deficiency. Finally, the inverse relationship between 3H-TdR incorporation into DNA and folate status observed in this study has been similarly described in cells with either in vivo or presumed in vitro acquired folate deficiency.

The biochemical basis for these observed changes in pyrimidine metabolism has been attributed to altered thymidylate synthetase activity consequent to intracellular folate deficiency, but this proposal is currently disputed. The in vitro model for folate deficiency and megaloblastic maturation reported here will allow for the ordered probing of these biochemical events, as it obviates the vicissitudes of obtaining deficient cells or the uncertain specificity in the use of metabolic inhibitors, such as methotrexate or 5-fluorouracil with normal cells.

Finally, our findings have clear implications for cytogenetics, for the description of these fragile sites on human chromosomes that are dependent on the type of culture medium employed in lymphocyte blastogenesis may in fact be a simple expression of the folate content of the medium.

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