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3 March 1967

Lactic Dehydrogenase and Metabolism of Human Leukocytes in vitro

Abstract. *During transformation and division of lymphocytes in culture, the lactic dehydrogenase isozymes migrate increasingly toward the cathode. With extension of the time in culture, the mitotic index declines, and the isozyme pattern reverts to dominance of those bands that move toward the anode, despite the cellular tendency to anaerobic metabolism. These findings suggest that synthesis of the more slowly migrating lactic dehydrogenase isozymes in this system is related to mitotic activity, and not to the aerobic or anaerobic conditions of cell culture.*

Primary cultures of human leukocytes provide a rapidly dividing and metabolically active tissue for the investigation of isozyme synthesis. Under stimulation with phytohemagglutinin (PHA), leukocytes in short-term, 72-hour cultures undergo an orderly series of morphological changes, beginning with degeneration of the granulocytes, and followed by transformation and mitosis of lymphocytes (*1*). There is an early increase in RNA synthesis (*2*) and in the phosphorylation and dephosphorylation of nuclear proteins (*3*). Together with the fact that the rate of protein synthesis is increased after stimulation with PHA, these findings have been interpreted as indicative of gene activation before or during mitosis (*3*).

Genetic control of the synthesis of the heart (H) and muscle (M) polypeptides of lactic dehydrogenase (LDH) is now well established (*4*). It has also been suggested that the bands of LDH that migrate towards the cathode are associated with conditions of relative anoxia and anaerobic metabolism, while the isozymes on the anode side predominate under conditions of higher oxygen tension and aerobic cellular metabolism (*5*). In our study of leukocytes stimulated with PHA, the relationship between transformation and cell division and the activity of a specific isozyme system was examined. During 168 hours of culture, sequential observations were also made on the energy metabolism of the cells.

Human leukocytes from normal volunteers were cultured, under stimulation of phytohemagglutinin, in tissue-culture medium 199 with a volume of fetal bovine serum equal to that of the cell suspension. From 6×10^6 to 8×10^6 cells were inoculated into each culture, and cultures were incubated at 37°C for 24, 48, 72, 96, or 168 hours. Cells were harvested, washed three times in isotonic saline, and homogenized in 0.5 ml saline, at room temperature, at 2500 rev/min, with a Teflon tissue homogenizer.

Analyses of glucose, pyruvic and lactic acids, and α-ketoglutaric acid were performed on samples of the medium. Electrophoresis on agar gel, and determinations of total lactic dehydrogenase activity and total protein were performed on cell homogenates. Five microliters of cell homogenate were used for each electrophoresis. The concentration of glucose was determined with a modification of Folin's micromethod (*6*); that of pyruvic acid, according to Friedmann and Haugen (*7*); and that of lactic acid, according to Huckabee (*8*). Determinations of the amounts of α-ketoglutarate were done on the basis of Shimizu's quantitative technique for extraction of its 2,4-dinitrophenylhydrazine derivative into xylol (*9*). The assay for the total activity of lactic dehydrogenase was adapted for cell homogenates from the procedure originally described by Wroblewski for reduction of pyruvic acid in the presence of reduced nicotinamide-adenine dinucleotide (NAD) (*10*). Determinations of total protein were done according to the Lowry technique (*11*). Electrophoresis of cell homogenates was carried out on agar gel at 4°C with sodium lactate as the substrate, NAD as the coenzyme, and phenazine methosulfate as the electron carrier. A purple band was produced at the sites of LDH activity, by reduction of nitro blue tetrazolium to formazan. The following results are derived from studies of 95 cultures from 16 subjects.

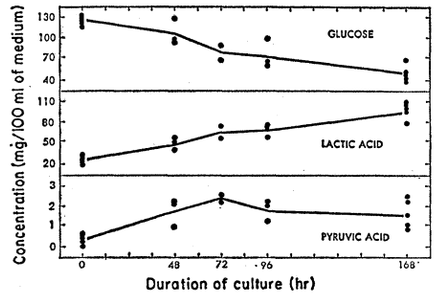

Fig. 1. Glucose and lactic and pyruvic acids in the medium of leukocyte cultures. Each point represents a single determination from a culture assayed at the indicated time. The curves are derived from plots of the mean values obtained.

In the first 72 hours of culture, the concentrations of pyruvic and lactic acids rose, while that of glucose in the medium fell. Continued culture of these leukocytes resulted in a further increase in the concentration of lactic acid, with a precipitous decline in available glucose in the medium by 168 hours (Fig. 1). The concentration of pyruvic acid, however, appeared to reach a plateau, after increasing in the first 72 hours of culture.

In all cultures, total activity of LDH had increased by at least 100 percent by 72 hours of culture, the time of maximum mitotic activity, with mean values ranging from 1130 Wroblewski units per milliliter of cell homogenate before culture, to 2450 units at 72 hours, and 2200 units at 168 hours. Values for total protein fell and stabilized, during the first 72 hours of culture, from a mean of 3.0 mg/100 ml of homogenate before culture, to 1.9 mg/100 ml of medium at 72 hours. This decrease in total protein early in culture reflected degeneration of the granulocytes, the largest cell population inoculated into culture. Thus, the specific activity (Fig. 2) defined as the number of units of LDH per milligram of protein rose most significantly before mitosis and during active mitosis.

The shift in isozyme pattern normally seen is shown in Fig. 3, with the increase in LDH-3 and LDH-4 at 72 hours indicating a decrease in H-subunit activity and an increase in the activity of M subunits (*12*). In the first

48 hours of culture, the isozyme pattern closely resembled that observed in the cells before culture, and the percentages of H and M subunits similarly did not significantly change. The subsequent increase in M subunits thus occurred with the increase of cells in active division, between 48 and 72 hours, and not with the initial increase in transformed cells, seen between 24 and 48 hours. While the decrease in H subunits, and therefore the increase in M subunits, was consistent for all cultures during the initial mitotic cycles, at 168 hours there was some variation. Three of the nine cultures studied at 168 hours showed a continued decrease in LDH-1 and LDH-2 with a 1 to 5 percent decrease in H-subunit activity, relative to the values at 72 hours. The remaining six cultures, however, showed increased activity of isozymes near the anode, with a 6 to 22 percent increase in H subunits. This variation was present despite a relative constancy of mitoses and transformed cells at this time. The mean values for H-subunit activity in the nine sets of cultures were 70.4 percent before culture, 60.1 percent at 72 hours, and 67.3 percent at 168 hours.

The mitotic characteristics of the cell population in culture also were changing. In the first 24 hours, the number of cells in active division per 1000 cells examined (the mitotic index) was less than 5. Many of the cells seen at this stage were nonviable granulocytes. By 48 hours, the mitotic index had risen from 5 to an average of 20 and transformed lymphocytes and small lymphocytes made up virtually the entire population. At 72 hours of culture, the mitotic index reached a peak of 70 to 80, with a continued increase also in the percentage of transformed, immature lymphocytes from 10, at 48 hours, to 15. By 168 hours, the mitotic index had fallen to 20 or less in all cultures, with histologic examination revealing mixed colonies of small lymphocytes and transformed cells in approximately equal proportions. There was little or no evidence of cellular degeneration, such as vacuolization of the cytoplasm or nuclear pyknosis, after the first 24 hours of culture. The continuous uptake of glucose from the medium and the increasing concentrations of lactate in the medium support the histologic evidence of a viable lymphocyte cell population throughout the 168-hour culture period.

When leukocytes were cultured in the absence of a mitogenic stimulant

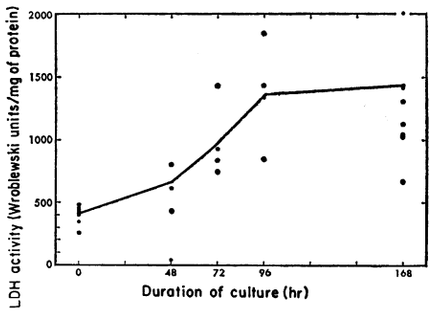

Fig. 2. Specific activity of lactic dehydrogenase in cultured human leukocytes. With degeneration of the large granulocyte population, the decrease in protein per milligram of cell homogenate and the concomitant increase in total LDH activity produced a rise in specific activity throughout the cell culture, but this increase was maximum between 48 and 96 hours.

in tissue culture medium 199 and the patient's own serum, the mitotic index remained below 5 throughout the 168-hour culture period, and histologically there was continuous evidence of cellular breakdown. The majority of lymphocytes, however, did appear intact. In contrast to cultures of dividing cells, cultures of essentially nondividing cells showed: (i) mean values for total LDH activity of 1100 units before culture, 1040 units at 72 hours, and 980 units at 168 hours; and (ii) mean values for H-subunit activity of 69 percent before culture, 74 percent at 72 hours, and 74 percent at 168 hours. The absolute increase in LDH activity seen in cells stimulated with PHA was caused largely by the increase in the activity of M subunits, and the failure of LDH activity to increase in cells not stimulated with PHA was due largely to the failure of the activity of M subunits to increase.

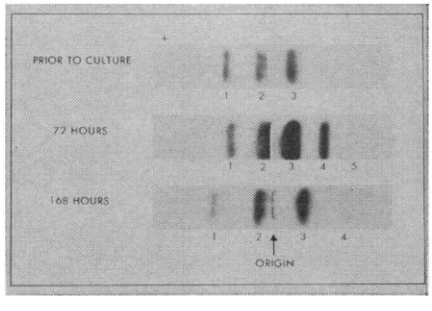

Fig. 3. Patterns of lactic dehydrogenase isozymes from leukocytes before culture and at 72 and 168 hours of culture. The bands that migrated to the anode in this system of agar-gel electrophoresis were LDH-1 and LDH-2; those that migrated to the cathode were LDH-3, LDH-4, and LDH-5.

In the cultures of stimulated cells, the markedly increased concentrations of pyruvic and lactic acids found at 72 hours raised the possibility of a shift in the metabolic processes of the cells toward anaerobic glycolysis with increasing time in culture. Assays of α-ketoglutaric acid in the medium showed a 5- to 15-fold increase in its concentration in the first 72 hours of culture, increasing from 0.1 to 0.2 mg/100 ml of medium prior to culture to 1.0 to 1.5 mg/100 ml of medium at 72 hours. However, between 72 and 168 hours, the amounts either decreased or remained constant, suggesting, as supported by the continuing rise in lactate, that anaerobic glycolysis had become a significant energy source.

We evaluated the effect of increased concentrations of pyruvic or lactic acids early in culture to determine whether these substrates alone could induce formation of bands that migrate toward the cathode. Pyruvic acid (0.14 to 1.44 mmole per liter) was added to these cultures in single or multiple doses, at 24-hour intervals, for the first 3 days. This range encompassed maximum pyruvate concentrations found in the leukocyte cultures. Similarly, sodium lactate (3.0 to 12.0 mmole per liter) was added to other cultures, and as with the addition of pyruvic acid, no alteration in the normal sequence of isozyme synthesis was produced. At concentrations above these, cell viability was impaired, and the cultures failed.

To test for enzyme leakage during granulocyte degeneration and subsequent division of lymphocytes, we performed gel electrophoresis and assays for total LDH activity on culture medium concentrated in Sephadex G200. Before culture, and at 24, 48, 72, and 168 hours of culture, trace amounts of those isozymes that migrate to the anode were detectable on electrophoresis. Before culture, the total activity of LDH in the medium bathing the cells was less than 1.0 percent of that of the cell homogenates, reflecting only the LDH activity of the added fetal bovine serum. From 24 hours of culture, as LDH activity in the homogenate rose, the total LDH in the medium also rose, being consistently 10 to 20 percent of the cellular amount for all cultures. Further, analyses of enzyme in the cell washings obtained before homogenization revealed no detectable activity at any time.

With 60 to 70 percent of the inoculated cells dying in the first 24 hours

of culture and with a maximum of 7 to 8 percent of the lymphocytes in active mitosis at any one time, the 10 to 15 percent of lymphocytes which appeared as transformed cells before, and after, 48 hours was doubtless metabolically significant. However, since the isozyme pattern changed maximally with the burst in actively dividing cells between 48 and 72 hours, it seems likely that mitosis rather than the period before mitosis, or transformation, was largely responsible for the activation, or de-repression, of the genetic locus controlling synthesis of M subunits. With the observed decline in cell division at 168 hours, this locus may have again become inactive, the isozymes tending to return to the pattern seen before culture and during early transformation.

Cultures of leukocytes are heterogeneous with respect to cell type and the relationship of cells to division. While more than 90 to 95 percent of the cells are lymphocytic in origin, the contribution of the remaining 5 to 10 percent of cells to both isozyme synthesis and energy metabolism is unclear. Further, at any given time beyond 48 hours, the cultures consist of nontransformed, transformed, and actively dividing cells. This heterogeneity and the asynchronous cell division preclude a definitive statement of when in interphase (before, during, or after DNA synthesis) or mitosis the isozyme shift took place.

The application of this study to cell functioning in vivo is unclear. The activity of genes under the stimulus of a potent mitogenic agent and in the presence of a synthetic medium for support of cell division precludes direct comparison with cells in the soma. However, it seems reasonable to conclude that isozyme synthesis does not simply depend on an adaptation of cells to a changing biochemical environment or to a changing metabolic pathway.

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12. From density tracings of the electrophoretic patterns obtained on the gel, the percent of each LDH band was calculated, and the distribution of H and M subunits was estimated. The proportions of H and M were assumed to be 4 : 0, 3 : 1, 2 : 2, 1 : 3, and 0 : 4 in LDH bands 1 to 5, respectively.
13. A.D.B. is a Surgeon, U.S. Public Health Service, National Center for Radiological Health, on assignment to the Atomic Bomb Casualty Commission, an agency of the U.S. National Academy of Sciences–National Research Council and the Japanese National Institute of Health.

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Cell-Bound Immunity to Autologous and Syngeneic Mouse Tumors Induced by Methylcholanthrene and Plastic Discs

Abstract. *A colony-inhibition assay was used to demonstrate specific immunity in vitro against syngeneic mouse sarcomas induced by methylcholanthrene or implanted Dictabelt plastic discs. An immunity to methylcholanthrene-induced tumors could also be demonstrated with lymphocytes derived from the autologous primary tumor-bearing host 3 to 8 days after removal of the primary tumor.*

Most experimentally induced neoplasms have specific tumor antigens which can be detected with the isograft-rejection technique (*1*). Tumors induced by methylcholanthrene (MCA) and other chemical carcinogens or by implantation of plastic films contain antigens which are individually distinct for each tumor when the tumors are also induced by the same agent (*2*). They differ in this respect from virus-induced tumors, which have specific antigens common to all neoplasms induced by the same virus (*1*).

Many methods have been used to demonstrate tumor specific antigenicity in vitro (*1*). We consider the recently introduced colony-inhibition (CI) assay to be of particular interest (*3*), since it probably reveals the same antigens as the isograft-rejection technique. It has been used to detect both cell-bound and humoral immunity to neoplasms induced by adenovirus 12 (*4*) and by the polyoma virus (*3*). We now report on data from a colony-inhibition assay of cell-bound immunity to specific antigens in mouse sarcomas induced by MCA and by plastic discs. These types of tumors were chosen, since they originate only at the place of carcinogen implantation and can therefore be easily removed. This makes it possible to investigate immunity to such tumors in the autologous host which may provide a model for similar studies with human material.

Sarcomas were induced in C3H male mice implanted with 3-methylcholanthrene pellets; they were individually numbered (Table 1). Three sarcomas were induced in BALB/c females, implanted with blue Dictabelt plastic discs (*5*). All mice were derived from a single line inbred colony and were skin-compatible.

Primary tumors were removed and used for explantation in vitro and transplantation to syngeneic mice. The explanted cells were propagated in vitro for 2 to 60 days before CI assays which were performed on tumor cells plated in 50-mm Falcon plastic petri dishes (*3*). Approximately 24 hours after plating of the tumors when the cells had attached to the petri dish surfaces, the medium was decanted, and 0.3 ml of either Difco phytohemagglutinin (PHA), diluted 1:15, or phosphate-buffered saline (PBS) was added to the petri dishes. Immediately afterward, 0.5 ml of a suspension of lymph node cells in Eagle's minimal essential medium (MEM) was also added. Two or three doses of lymphocytes were tested: 5×10^6, 2×10^6, and 10^6 cells per petri dish. After the cells were incubated for 45 minutes, each petri dish received 4 ml of MEM containing heat-inactivated fetal calf serum (15 percent). The dishes were then incubated at 37°C for 3 to 4 days in an atmosphere of 5 percent CO_2 and air, the colonies were stained with 0.1 percent crystal violet, and the number of colonies formed by the target cells was counted. The lymphoid cells did not form any colonies under the conditions of the experiments.

The lymphoid cells tested on the target cells were derived from axillary, cervical, inguinal, and mesenteric nodes.