Intracellular Protein Breakdown in the L1210 Ascites Leukemia

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SUMMARY

The endogenous catabolism of the cell protein of the L1210 mouse ascites tumor has been estimated from its in vitro breakdown to free amino acid after pulse labeling in vivo with leucine-1-14C. Two general protein classes are distinguishable according to their breakdown: an unstable population representing one-fifteenth of the cell protein and with an average half-life of several hours, and the stable remaining protein with a uniform breakdown one-fifth the cumulative rate of the unstable class. Treated as a parameter of growth, the cumulative turnover of the unstable class requires the expenditure of 5% of the biosynthetic capacity of the cell per hour, and comprises one-third of the total cellular protein turnover of 1.5%/hour.

None of a variety of inhibitory agents administered in vivo or in vitro selectively stimulates or inhibits the cumulative breakdown rate. However, the selection of proteolysable cellular substrates varies with the physiologic state of the cell. After treatment of the tumor with chemotherapeutic agents, breakdown is expanded to a broader spectrum of cellular substrates than are proteolysed in growth.

INTRODUCTION

A number of estimates have been made, both in vivo and in vitro, of the breakdown of endogenous protein by tumors (2, 6-8, 13, 14). A uniform breakdown of approximately 1% of the cellular protein per hr has been reported to be general for mammalian cell cultures irrespective of origin (5). Measurement of breakdown of tumor protein in vivo, which is experimentally more difficult, has not been made with comparable precision (8). Past estimates in general have relied on uniform decay rates, and there is little assurance that they are maximal. A greater, more inclusive valuation of breakdown will be described for the L1210 mouse ascites leukemia which takes into consideration the marked heterogeneity of the substrate populations involved in turnover as well as the variation in substrate choice for breakdown that occurs with the physiologic state of the cell.

MATERIALS AND METHODS

DBA/2 male mice at the age of 2-6 months were inoculated i.p. with 10^6 to 10^8 ascitic L1210 cells. For drug treatment, 2 i.p. doses of 4',4''-dichlorobis(2-imidazolin-2-yl)terephthalanilide at 50 mg/kg, methylglyoxal bis(guanhydrazone) at 100 mg/kg, hydrocortisone acetate at 50 mg/kg, and 6-mercaptourine at 40 mg/kg were given on each of the last 2 days of tumor growth, the last dose 5 hr before harvesting. With methotrexate treatment, a single dose at 20 mg/kg was given 18 hr before harvesting. To label cell protein, 0.7 µg of L-leucine-1-14C containing 10^6 cpm was given i.p. 30 min before harvesting unless specified otherwise. Five to seven days after tumor implantation, non-bloody ascites containing a packed volume of 0.03 to 0.2 ml of cells were flushed out of the peritoneal cavity with several portions of saline solution generally containing carrier leucine. The cells were centrifuged and resuspended in 10 ml of Eagle's medium without serum, with the leucine level raised to 500 µg/ml.

To determine protein breakdown, the passage of label from cellular protein was followed into the carrier. Samples of 3 ml of the labeled cell suspension were shaken at 37°C under an atmosphere of air supplemented with 5% CO₂. Incubation was terminated by the addition of 0.2 ml of 50% citric acid, followed by boiling for 5 min. This treatment has been found to precipitate over 95% of the protein. The leucine-1-14C of the supernate was decarboxylated with ninhydrin and the evolved CO₂, which can be generated from leucine only as a free amino acid, was collected for counting as described previously (15). Protein was counted directly (15). Turnover is calculated as [(a÷fina - a÷inital)/b] X 100, where a is the activity in cpm evolved by decarboxylation and b is the total activity of the protein. Final values were obtained routinely after 1 hr of incubation. Only the intracellular events of breakdown and resynthesis will be connoted by protein turnover, to the exclusion of secretion as an additional site of cellular loss of protein. Modifications of the above procedures are specified in the text.

Subcellular fractions of the L1210 tumor were obtained by cell disruption with glass beads, followed by centrifugal fractionation (17). Cathepsin activity was determined with acid hemoglobin as the substrate (1). Protein was determined by the Lowry procedure (11).

RESULTS

The effective period of uptake of a single i.p. leucine-1-14C dose by the tumor is brief. A maximum incorporation that may reach 10% of the administered label is evident in the tumor protein at the earliest harvest attempted, 7 min after injection. If the labeling period had been prolonged and the protein totally labeled, the proteolytic rate ensuing would be that of an average cellular protein. However, the pulsed labeling used preferentially enters the most labile proteins of the cell in proportion to the need for their more frequent resynthesis. The resulting catabolism gives the cumulative rate of proteolysis as a parameter of current protein biosynthesis, and it proceeds at a rate of 6%/hr judging from the breakdown of recent label administered 30 min before.
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Chart 1. The course of in vitro catabolism of leucine-1-14C-labeled cellular protein of the L1210 ascites leukemia. Cells were harvested 30 min or 24 hr after i.p. isotope injection. Standard conditions of measurement were used except for the two values (X) where the cells were flushed with 1:1 DBA/2 blood serum:Ca++-free Hinger solution + 500 µg/ml leucine, and incubated without washing. Each preparation was pooled from 2 animals, and each determination was made in duplicate.

Hand (Chart 1). The initial value recapitulates the extent to which breakdown of the various protein populations, paired with their biosynthetic replacement, would occur during net growth. The rate did not vary among preparations of varying cell age and cell density. It is also unaugmented when manipulations are minimized by flushing the cells out of the peritoneal cavity into a medium of diluted blood serum (Chart 1). The initial rate is much higher but less constant than the decomposition of protein labeled 24 hr beforehand (Chart 1), indicating progressive substrate exhaustion even during the 2.5-hr period of observation. In the protein with the older label, preferred catabolic substrates evident in fresh labeling would now presumably be replaced with unlabeled leucine derived from the host. From Chart 2, the decrease from the high initial catabolic value is seen to be rapid, reaching within 9 hr a stable rate common to Eagle's value of 1% of the cell label/hr for totally-labeled cell cultures (5), and appears to be maintained indefinitely thereafter. The total decomposition that appears to be due to recycling of the most unstable cell material may be estimated from the area below the curve in excess of 1%/hr. By the time it is exhausted at 9 hr, it would not have used up more than one-third of the original cell label and it would represent a far smaller fraction of the total protein. Thus, although the average breakdown rate for the bulk of the protein, and presumably that for the totally-labeled protein of the cell does not differ from that of cell cultures (5), the cumulative turnover breakdown rate appears greater.

Chart 2. The course of breakdown of label remaining in the protein of the L1210 tumor during 4 days of growth after i.p. administration of leucine-1-14C at 0 hr. Breakdown was determined in vitro for 1 hr. Values at 1, 3, 24, 48, and 72 hr are averaged from 2 to 6 experiments, and the remaining values are of single experiments. In each experiment, duplicate values were determined, usually of pooled tumors.

To ascertain that the final breakdown in Chart 2 comes from the original proteins and is not reassimilated from the proteins of host serum, the tumor cells were transferred to a new host one day after labeling. After two additional days of growth, the catabolic rate was again found to be 1%/hr.

Uniformity of Cellular Labeling

The estimation of maximal breakdown rate is valid only if the leucine label is uniformly accessible to all subcellular sites of protein synthesis. Complete equilibration of amino acid labels with the cellular pool appears difficult to obtain in the liver (9) and in the Walker carcinosarcoma 256 (2, 14). Neither of these systems in comparison to the present one, however, incorporates amino acid so directly without the intervention of circulatory transport, and at the same time comprises a uniform and rapidly-growing cell population. From Table 1, it is evident that with a 1000-fold increase in amino acid level, pool equilibration is being approached. The administered leucine at the high level must be supplanting a substantial part, if not all of the normal supply of the host's amino acid for a significant part of the incorporation period, without any change resulting in the breakdown rate of the protein. The uniformity of incorporation of labeled leucine at the standard dosage is also evident from its intracellular distribution. The specific activities of the nuclear, mitochondrial, microsomal, and soluble proteins have been found to be within 8% of that of the total cellular protein.

Effects of Inhibitors on Catabolism

In preliminary experiments a variable stimulation of proteolysis was found when the L1210 tumor was treated with growth-inhibitory drugs such as methotrexate or dichloro-bis(imidazolino)terephthalanilide. However, the stimulation, as shown in detail for methotrexate treatment in Table 2, is confined to the stable, not the cumulative proteolytic rate. When growth is arrested, there is no effect on the maximal catabolism (cumulative turnover) at 1 hr after labeling, but at 3 hr the residual rate does not fall off as rapidly in the treated cell, the breakdown being heightened for a while at about twice comparable normal values (Ex-
Effect of Growth Inhibition by Methotrexate on Endogenous Protein Catabolism of the L1210 Leukemia

Experiment 1, Table 2. The breakdown of normal protein labeled before drug treatment is also heightened somewhat (Experiment 2, Table 2). Thus the inhibited cell catabolizes its protein more indiscriminately, but not to a greater extent than the growing cell. In support of this evidence of undeviating catabolism the labile proteins are pulse-labeled in new cell material representing, in 1 hr, an 8.3% growth expansion of tumor (generation time = 12 hr) and a replacement of 1% of the stable protein. The breakdown rate of the unstable class would be 5% of 9.3% or about 0.5%/hr in terms of total cellular protein. This and the stable breakdown comprise a 1.5% total turnover rate in all cell proteins, the turnover of which is half completed about 3 hr after labeling (Chart 2). Turnover of this class would be rated at 5%/hr in vivo and for its release in vitro may not appear in accord with the steady breakdown rate of stable protein had been relied on.

Although virtually all of the internal proteins of the mammary cell are probably susceptible to catabolism (3) and therefore require some turnover replacement during growth, much of this activity is due to repeated synthesis in a class of minor labile proteins, the turnover of which is half completed about 3 hr after labeling (Chart 2). Turnover of this class would be rated at 5%/hr of the growth incorporation, which together with the breakdown of the stable class gives the cumulative rate of 6%/hr. The labile proteins are pulse-labeled in new cell material representing, in 1 hr, an 8.3% growth expansion of tumor (generation time = 12 hr) and a replacement of 1% of the stable protein. The breakdown rate of the unstable class would be 5% of 9.3% or about 0.5%/hr in terms of total cellular protein. This and the stable breakdown comprise a 1.5% total turnover rate in all cell proteins. Since the unstable proteins contribute 1/ of the total leucine requirement (Kg) with the stable proteins contributing 3/ of the total leucine requirement (Kg).

**TABLE 2**

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Schedule of administration</th>
<th>Tumor catabolism</th>
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<tbody>
<tr>
<td></td>
<td>Methotrexate (hr)</td>
<td>Leucine-1-¹⁴C (hr)</td>
</tr>
<tr>
<td>1</td>
<td>0 and 6</td>
<td>24</td>
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<td>49</td>
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a. The dose used (20 mg/kg) is effective against the tumor at 24 hr, as judged by reabsorption of ascites fluid and by 60% or more inhibition of leucine-1-¹⁴C incorporation into protein.

b. Average of duplicate values for 1-3 pooled tumors.

**DISCUSSION**

Although virtually all of the intracellular proteins of the mammary cell are probably susceptible to catabolism (3) and therefore require some turnover replacement during growth, much of this activity is due to repeated synthesis in a class of minor labile proteins, the turnover of which is half completed about 3 hr after labeling. Turnover of this class would be rated at 5%/hr of the growth incorporation, which together with the breakdown of the stable class gives the cumulative rate of 6%/hr. The labile proteins are pulse-labeled in new cell material representing, in 1 hr, an 8.3% growth expansion of tumor (generation time = 12 hr) and a replacement of 1% of the stable protein. The breakdown rate of the unstable class would be 5% of 9.3% or about 0.5%/hr in terms of total cellular protein. This and the stable breakdown comprise a 1.5% total turnover rate in all cell proteins. Since the unstable proteins contribute 1/ of the total cell protein, they would comprise 1/ of the total cell protein.

When growth becomes inhibited, the estimation of turnover proteolysis as a function of current protein synthesis is particularly meaningful in showing that cumulative catabolism is unaffected in rate, but becomes more generalized, involving a broader protein spectrum, as indicated by the persistence of a greater amount of residual proteolytic activity in old protein after the initial maximum is spent. A spurious appearance of pronounced catabolic stimulation would have been given if the steady breakdown rate of stable protein had been relied on.

The use of different experimental conditions for uptake of label in vivo and for its release in vitro may not appear in accord with the ideal of a steady-state condition for evaluating the true extent of protein turnover. However, the procedure may actually be experimentally superior to a wholly in vitro system. The removal of the original label by rapid and exhaustive cell washing would be most likely to introduce serious experimental effects of its own. This is obviated in the present in vivo administration by the efficiency of the host as an amino acid trap in flushing the label out under the same conditions in which it was introduced. The high initial rate of in vitro proteolysis of freshly-labeled protein could be due to its inability to become normally integrated in the...
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Intracellular protein breakdown in L1210 ascites leukemia cell when its environment is altered. If so, the lability might have been expected to lessen in Chart 1 when the harvesting and the change in milieu become greatly minimized, or to increase on the other hand in any of the in vivo or in vitro drug treatments. This does not happen, even though a growth-inhibitory effect is evident from an increase in the average breakdown rate. The cumulative turnover rate appears to be fixed also in the sense that it is uninfluenced by variation in tumor age or in cell concentration even beyond the limits reported.

Most of the cell protein is slowly digested, seemingly in bulk without evidence of pronounced discrimination in turnover. For the especially labile cell protein, a number of obvious functions may be surmised. Much of the amino acid requirement of rapidly proliferating tumors is derived from the host's serum (2), and its cellular uptake and digestion by pinocytosis would entail turnover of lysosomal and vacuolar structures (4). In addition, the nuclear histones and the mitotic apparatus might be expected to comprise major protein components in a cell with a small cytoplasm and could require complete and extensive destruction (9, 12) during rapid growth.

Two points of similarity may be cited between this mammalian system and the system of protein turnover previously investigated in Escherichia coli (15). First, the cumulative rate of protein catabolism of the cell is unvarying and unaffected by the physiologic status of the cell. Second, during growth arrestment, a broader population of cellular substrates are catabolized than during growth. This may be because the normally preferred substrates are exhausted and cannot be easily regenerated without growth, or alternatively, since much of the cell protein may not be functioning in the absence of growth, it may be allosterically modified towards greater susceptibility to catabolism.

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REFERENCES


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