In Vitro Incorporation of C^{14}–DL-Leucine into Normal and Leukemic White Cells

By W. H. Baker, P. C. Zamecnik and M. L. Stephenson

IN 1911, Grafe demonstrated that human leukemic leukocytes possessed an oxidative metabolism.[1] Since that time, many comparative studies on normal and leukemic human leukocytes have revealed major quantitative differences in their biochemical and enzymatic constituents.[2-5] Details of these differences may be found in the recent reviews of this subject by Valentine.[6,7]

Previous experiments done in this laboratory showed that the in vitro incorporation of C^{14}-leucine or C^{14}-alanine into the white blood cell proteins was of the order of six times greater in cells from patients with acute leukemia, as compared with normal white cells. White cells in chronic leukemia, both myelogenous and lymphatic, showed incorporation rates which were only slightly higher than normal cells.[8] More recently, Weisberger et al. studied the uptake of S^{35}-L-cystine and S^{35}-L-methionine by normal and leukemic cells in vivo and by leukemic cells in vitro.[9,10] In vivo studies revealed that leukemic cells had a higher uptake of these two amino acids than did the normal white blood cells, and led to the use of selenium cystine in therapeutic trial for acute leukemia.[11] Since the in vitro technic measures an active metabolic process, the object of the present study was to utilize this method to follow the in vitro survival time of both normal and leukemic white cells.

Materials and Methods

White Cell Preparations

Normal white blood cells were separated from whole blood by a falling film centrifuge and were relatively free of red blood cells. These cells were obtained through the courtesy of Dr. James L. Tullis. The harvested white cells were resuspended to approximately 15,000 to 20,000 cells per cu. mm. in modified Ringer's solution, as previously described by Tullis.[12]

Aliquots of 1 ml. of this suspension were placed in siliconed vials, one inch in diameter. Approximately 2.5 μM DL-leucine-1-C^{14} containing 200,000 CPM were added to each vial. The final volume was 1.2 ml. Incubation was carried out at 37 C. in 95% oxygen-5% carbon dioxide for varying lengths of time.

DL-leucine-1-C^{14} was synthesized for us by Dr. Robert B. Loftfield of this laboratory.

In the studies which compared the incorporation of labeled amino acids into proteins from control and leukemic white blood cells, the white cells were separated from the red cells by the fibrinogen flotation method of Minor and Burnett.[13] Four hundred and fifty to five hundred ml. of whole blood were collected through siliconed needles and tubing into plastic bags, in order to decrease the destruction of the cells. After incubation with fibrinogen (approximately 0.3%), the suspension of white blood cells in plasma was centrifuged for

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five minutes at about 500 × g. Most of the supernatant was drawn off and the cells resuspended to the desired concentration in the medium, which contained 0.2 mg. heparin/ml. Control cell suspensions of this series contained some red blood cells. The red blood cells did not incorporate the radioactive amino acid into their protein.

Treatment of Cells After Incubation. After incubation, the cells were washed twice with 10 ml. of 0.9% sodium chloride. Ten ml. of 4% trichloracetic acid were then added to precipitate the protein. This protein precipitate was washed twice with 10 ml. of 4% trichloracetic acid, and treated a third time with 10 ml. of hot 4% trichloracetic acid for 20 minutes at 90 C. The precipitate was next washed with 10 ml. of warm absolute ethanol, followed by a 2:2:1 mixture of warm ethanol, ether, and chloroform. The precipitate was then resuspended and homogenized in a known volume of acetone. Since the amount of protein was often too small to weigh accurately, the ninhydrin procedure of Moore and Stein was carried out on small aliquots of hydrolyzed suspension in order to determine the amount of protein present.[14] The remaining protein was filtered onto Whatman #50 paper for counting. A thin window Geiger-Muller tube and scaling circuit, with a counting efficiency of approximately 15 per cent, were used to count the samples. In most instances, duplicate determinations were carried out.

Results

Figure 1 demonstrates the effect of varying the incubation time on the rate of incorporation of DL-leucine-C^{14} into the protein of normal white cells. There is a steady increase in the amount of leucine incorporated over a period of two hours.

Figure 2 shows the effect of adding increasing concentrations of DL-leucine in order to determine the concentration at which the system becomes "saturated" and beyond which no further addition of leucine increases the amount incorporated. The figure shows that with cells incubated both in plasma and in gelatin, the curve begins to plateau around 0.003M DL-leucine. Therefore, most experiments were run near this leucine concentration.

Incubation in nitrogen effectively decreased but did not completely inhibit the incorporation. This is consistent with the findings of Weisberger.[10] The addition of 5×10^{-4} M dinitrophenol likewise decreased the rate of incorporation.

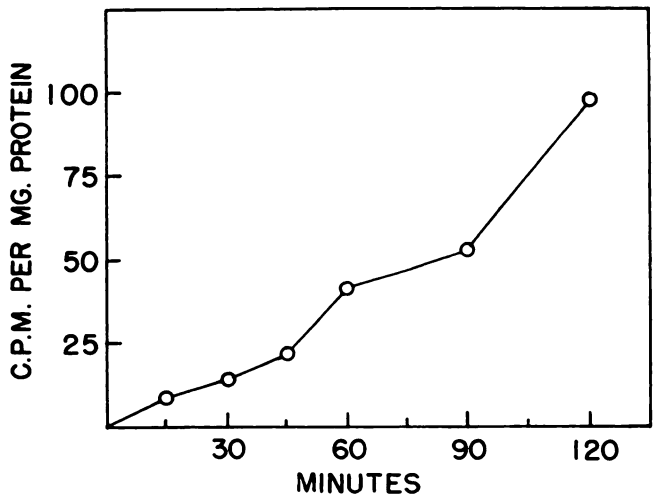

FIG. 1.—Time curve of incorporation of C^{14}-DL-leucine into normal white cell protein. Each flask contained 0.5 mM C^{14}-DL-leucine, containing 120,000 CPM/ml.

A comparison of incorporation of both DL-alanine-1-C^{14} and glycine-1-C^{14} into the protein of normal cells and leukemic cells had been made earlier. We had found that after $2\frac{1}{2}$ hours' incubation, in chronic leukemia, both myelogenous and lymphatic, the rates were only slightly higher than in normal cells; however, in acute leukemia, the uptake was several times greater than into the normal cells.[8]

Figure 3 shows a time curve of both normal cells and those from a patient with chronic myelogenous leukemia. The leukemic cells continue incorporating for at least five hours, whereas the control cells tend to plateau after two hours.

A study was carried out to determine the effect of storage at 3 C. on the metabolic activity of white cells, as measured by amino acid incorporation. Since incorporation was fairly linear for two hours at 37 C., it was decided to use this time period for incubation of the previously stored cells.

Figure 4 shows that when cells were incubated after storage, in both control and leukemic cells, there was a decline in the amount of leucine incorporated and that after four days of storage, very little leucine was incorporated. This figure correlates quite well with metabolic studies done by Tullis on phagocytosis, oxygen utilization, and dye permeability as measures of survival of normal white cells.[4]

Attempts were made to stimulate the incorporation of the labeled amino acid into the cell protein and/or to prolong the survival time. For these studies

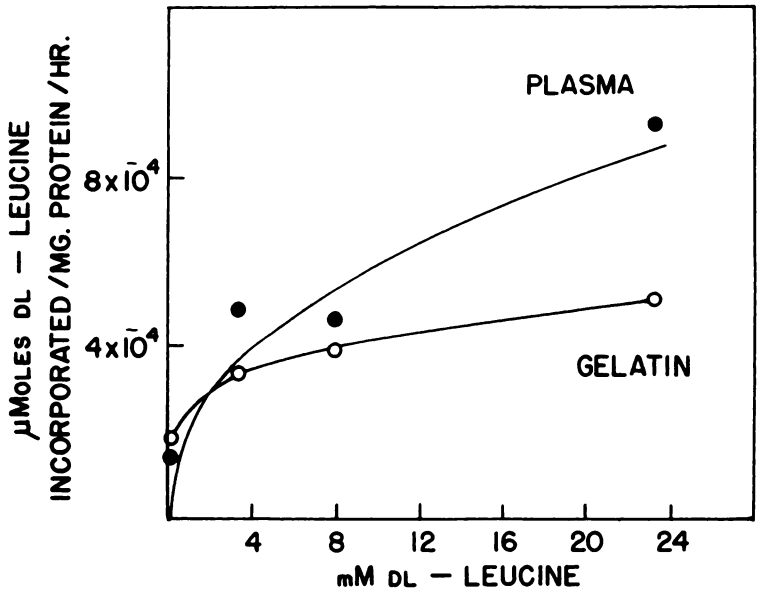

FIG. 2.—Effect of increasing concentrations of leucine on the incorporation of leucine into white cell protein. Each flask contained 330,000 CPM C^{14}-DL-leucine/ml.

μM DL-leucine incorporated per mg. per hour:

$$\frac{\text{CPM/mg. protein} \times \mu\text{M leucine in flask}}{\text{Total CPM in flask}}$$

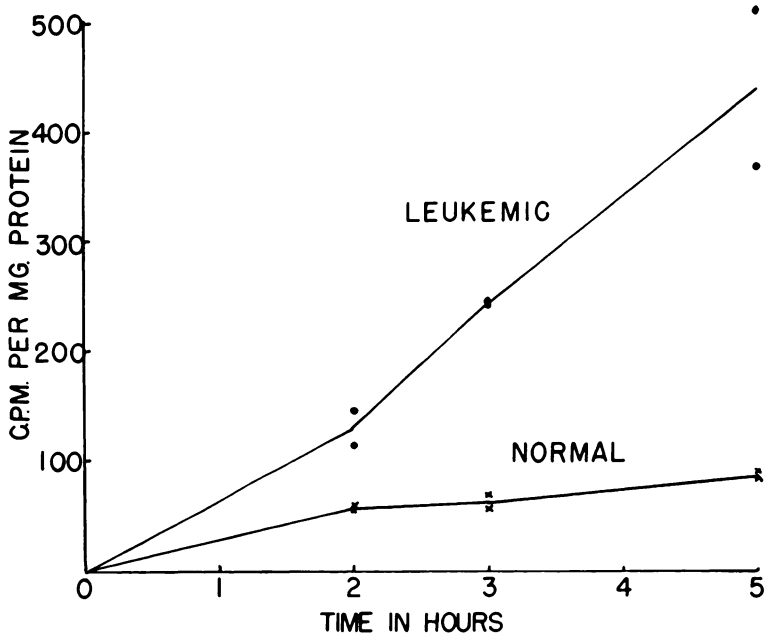

FIG. 3.—Time curves of incorporation of C^{14}-DL-leucine into normal and leukemic white cell protein. Each flask contained 4.0 mM C^{14}-DL-leucine containing 420,000 CPM/ml. The leukemic cells were from a patient with chronic myelogenous leukemia.

aliquots of the stored samples were taken at daily intervals and incubated with C^{14}-leucine for two hours at 37 C. Various lipids were added to the medium to see whether they might not "protect" the cell membrane and thus prolong the in vitro survival time of the cells. When 0.1 % hog liver lipid[15] was added to leukemic cells, there was a moderate decrease in the incorporation. There was no prolongation of survival time. Lecithin had no effect. Homologous plasma, heterologous plasma, and the supernatant from a homogenate of white cells were each added to control and leukemic cells at various intervals of one to three days in an effort to supply necessary factors which might prolong survival. In each case, there was no increase in the incorporation of C^{14}-leucine into the cell protein. Heterologous plasma decreased the uptake. This was probably due to the agglutination which occurred when the plasma was added.

The addition of 0.1 mg. of testosterone, hydrocortisone or cortisone did not change the incorporation rate.

Citrovorum factor had no effect on the incorporation rate of C^{14}-leucine into protein in leukemic cells, but incorporation into control cells was decreased. Aminopterin (0.1 mg.) had no effect. This may be due to the fact that the cells in vitro are already mature.

A similar in vitro experiment was carried out on platelets separated from human blood by the falling film centrifuge and suspended in gelatin at 4 C.* No incorporation of C^{14}-leucine into platelet protein occurred.

* Supplied through the courtesy of Dr. J. L. Tullis.

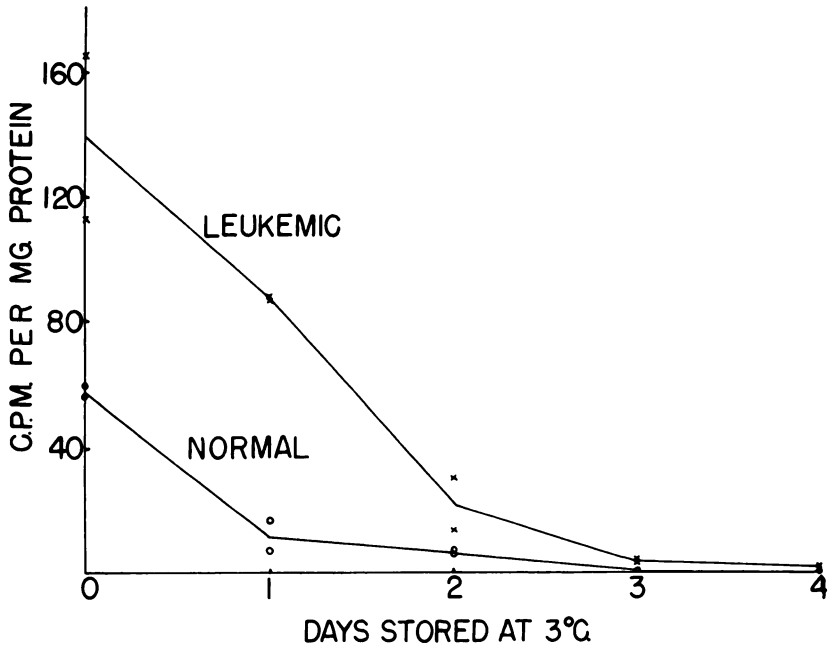

FIG. 4.—Effect of storage at 3 C. on the incorporation of C^{14}-DL-leucine into normal and leukemic white cell protein. Incubation was for two hours with 4.0 mM C^{14}-DL-leucine containing 420,000 CPM/ml. The leukemic cells were from a patient with chronic myelogenous leukemia.

DISCUSSION

These experiments support the evidence that normal and leukemic white cells incorporate C^{14}-labeled amino acids into proteins in vitro. Cells from individuals with leukemia are able to incorporate significantly greater amounts of the amino acid, and they are able to maintain this for a much longer period of time.

Since these represent an immature cell population, the increased incorporation may be a reflection of this immaturity, which all embryonic and cancer cells exhibit. Embryonic and regenerating livers, as well as liver tumors, all have a high rate of incorporation of labeled amino acids into protein.

The fact that the in vitro incorporation was not completely inhibited by dinitrophenol or nitrogen probably reflects the high rate of anaerobic glycolysis found to be present in white cells.

Despite the marked difference in ability to incorporate amino acids, both types of cells have the same in vitro survival time following storage at 3 C. The requirements which are necessary to maintain metabolic integrity are as yet undefined. It should be noted that although stored white cells may incorporate amino acids into protein in vitro, it is possible that they may nevertheless be irreversibly damaged in some other vital synthetic activity. In vitro viability as measured by this parameter is therefore not synonymous with in vivo viability. It is difficult to conceive of the cells being viable on reinjection, however,

if labeled amino acids are *not* incorporated into proteins under these conditions. From this point of view, the amino acid incorporation method can serve as a screening technic with which to test the relative effectiveness of new media and nutrilites proposed for trial in white cell storage procedures.

Attempts to prolong survival or to stimulate the initial incorporation rate have been unsuccessful. Part of this failure may be due to the inability of the various compounds used to penetrate into the cell.

Summary

Normal white blood cells and cells from patients with leukemia are able to incorporate C^{14}-DL-leucine into proteins in vitro. Cells from individuals with chronic myelogenous leukemia were able to incorporate significantly greater amounts of the amino acid, and they were able to maintain this incorporation for a longer period of time than the control cells.

Both types of cells had the same in vitro survival time of four days, following storage at 3 C.

The incorporation was partially decreased in the presence of dinitrophenol, as well as in an atmosphere of nitrogen.

Summario in Interlingua

Leucocytos normal e leucocytos ab pat entes con leucemia es capace in vitro a incorporar DL-leucina a C^{14} a in lor proteinas. Leucocytos ab individuos con chronic leucemia myelogene es capace a incorporar significativemente plus grande quantitates del amino-acido e a mantener iste incorporation durante plus longe periodos de tempore que cellulas de controlo.

Ambe typos de cellulas ha le mesme superviventia in vitro de quatro dies post immagasinage a 3 C.

Le incorporation esseva reducite in parte in le presentia de dinitrophenol e etiam in un atmosphera de nitrogeno.

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