with positive serum actually have the Japanese type of disease (ATL), or a disease closely related to it. In this regard, a study of the leukemic cell type in Japanese ATL will help elucidate the appropriate HTLV-susceptible cell.

The RNA tumor viruses have been linked to leukemias and lymphomas of animals since the pioneering studies of Gross (*16*). Subsequently, Jarrett and colleagues (*17*) were the first to associate the feline leukemia virus with a naturally occurring mammalian leukemia. Their studies, combined with those of Essex *et al.* (*18*), clearly demonstrated that FeLV causes feline leukemia. Other retroviruses cause some naturally occurring leukemias in chickens, wild mice, gibbon apes, and cattle (*19, 20*). The results described here are the first to our knowledge that demonstrate a human RNA tumor virus linked to a human neoplasia. This association and the known involvement of animal retroviruses in certain animal leukemias suggest that HTLV may be involved in causing these unusual human lymphoid malignancies.

MARJORIE ROBERT-GUROFF*
Laboratory of Tumor Cell Biology, National Cancer Institute, Bethesda, Maryland 20205
YOSHINOBU NAKAO
Kobe University School of Medicine, Department of Medicine, Kobe, Japan
KUNIHIRO NOTAKE
Aichi Medical University, Department of Microbiology, Nagoya, Japan
YOHEI ITO
Kyoto University Faculty of Medicine, Department of Microbiology, Kyoto, Japan
ANN SLISKI*
ROBERT C. GALLO
Laboratory of Tumor Cell Biology, National Cancer Institute, Bethesda, Maryland 20205

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* Present address: Department of Cell Biology, Litton Bionetics, Inc., Kensington, Md. 20895.

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Role for the Adenosine Triphosphate–Dependent Proteolytic Pathway in Reticulocyte Maturation

Abstract. *As reticulocytes mature into erythrocytes, organelles and many enzymes are lost. Protein degradation during reticulocyte maturation was measured by monitoring the release of tyrosine from cell proteins. Proteolysis in rabbit red blood cells was directly proportional to the number of reticulocytes and was low in erythrocytes. This process was inhibited by blockers of cellular adenosine triphosphate production and by agents, such as* o*-phenanthroline,* N*-ethylmaleimide, and hemin, which inhibit the soluble adenosine triphosphate–dependent proteolytic system. The breakdown of endogenous proteins in reticulocyte extracts was also inhibited by these agents and required adenosine triphosphate. Inhibitors of lysosomal function, however, did not affect proteolysis. Thus, the proteolytic system that degrades abnormal proteins also catalyzes the elimination of proteins during red cell development.*

When a mammalian reticulocyte matures into an erythrocyte, mitochondria, ribosomes, and many cytosolic enzymes disappear (*1, 2*). Since the loss of these cellular components from reticulocytes can occur in vitro (*3*), this process does not require other organs and apparently does not result from secretion of these structures out of the cell. Reticulocytes contain lysosomal proteases in membrane-bound form (*4*) and also a soluble, adenosine triphosphate–dependent (ATP-dependent) proteolytic system (*4–6*). One function of the nonlysosomal pathway is to hydrolyze proteins whose structures are highly abnormal (*4–8*), as may arise by mutation, biosynthetic errors, or incorporation of amino acid analogs. This pathway also seems to be involved in the breakdown of certain normal cell proteins; in adult hepatocytes, for example, the breakdown of short-lived proteins is not affected by inhibitors of lysosomal function such as chloroquine or leupeptin, which reduce the breakdown of more long-lived proteins in the same cells (*9*). In addition, in growing fibroblasts and myotubes, degradation of most cell proteins is not sensitive to inhibitors of lysosomal proteolysis, but does require ATP (*10, 11*).

We investigated whether the ATP-dependent or the lysosomal pathway is involved in the maturational breakdown of protein during reticulocyte development. Most studies of proteolysis in reticulocytes (*5–8*) have been limited to measurements of the breakdown of newly synthesized proteins. Since over 95 percent of the protein synthesized in these cells is hemoglobin, this approach is not useful for studying maturational loss of reticulocyte components, which were synthesized at earlier stages of the cell's development. To monitor their degradation, we used a fluorometric method to measure the net generation of free tyrosine from preexistent proteins (*12, 13*). We confirmed that there is no synthesis or oxidation of tyrosine in red blood cells (*1*) by establishing that no $^{14}CO_2$ or [^{14}C]hydroxyphenylpyruvic acid was produced from [^{14}C]tyrosine by rabbit reticulocytes and that no increase in tyrosine occurred when such cells were incubated with phenylalanine. Therefore, when protein synthesis is blocked by addition of cycloheximide, the tyrosine accumulating in the cells and medium results from the breakdown of cell proteins.

Reticulocyte production was induced in rabbits (males, 8 to 10 pounds) by subcutaneous injections of phenylhydrazine (25 mg/day) for 5 days. Two or more days after injections were stopped, sam-

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ples (~50 ml) of whole blood were centrifuged at 500*g* (4°C) for 10 minutes. The sedimented cells were then washed three times in cold Krebs-Ringer phosphate buffer, collected by centrifugation, and stained with new methylene blue to determine the reticulocyte number. The preparations studied contained 60 to 90 percent reticulocytes and no visible white cells. For measuring protein degradation, cells were suspended in four volumes of Krebs-Ringer phosphate buffer containing 10 m*M* glucose (except where noted) and 0.5m*M* cycloheximide. The cell suspension (5 ml) was incubated, with gentle shaking, in an open 25-ml flask at 37°C. Duplicate samples (1 ml) were removed periodically and added to equal volumes of cold 20 percent trichloroacetic acid. The samples were incubated at 4°C overnight and centrifuged, and the supernatant (1 ml) was assayed for tyrosine (*14*).

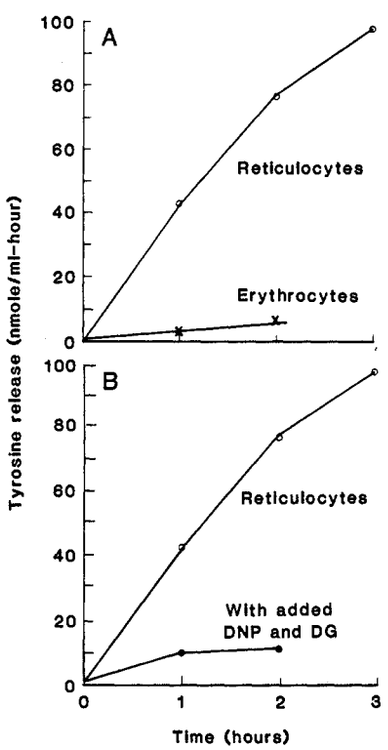

Fig. 1. Protein degradation in red blood cells measured by release of tyrosine from cell protein. Cell samples were prepared from phenylhydrazine-treated rabbits (reticulocytes) or controls (erythrocytes) and incubated as described in the text. The amount of acid-soluble tyrosine in cells and in the medium was determined at the times indicated, and the amount present initially was subtracted from each value. Before incubation, reticulocytes contained, on the average, 22 nmole of tyrosine per milliliter of packed cells. (A) Comparison of the rates of tyrosine production in a preparation containing 95 percent reticulocytes and an erythrocyte preparation (< 2 percent reticulocytes). (B) Effects on protein degradation in reticulocytes of inhibitors of ATP production, 2,4-dinitrophenol (*DNP*) (0.2 m*M*) and 2-deoxyglucose (*DG*) (20 m*M*).

Protein degradation in reticulocytes was monitored by measuring tyrosine production in the presence of cycloheximide. This process was linear for about 2 hours (Fig. 1), and then its rate decreased. During the incubation, intracellular tyrosine remained constant while tyrosine accumulated in the medium. Acid hydrolysis of the protein in a preparation containing 95 percent reticulocytes (255 ± 11 mg of protein per milliliter of packed cells) and subsequent determination of the tyrosine content (0.067 ± 0.007 nmole per microgram of protein) indicated that 0.55 percent of the cell protein was degraded per hour. This rate is less than the mean rate of protein breakdown observed with other mammalian cells (1 to 2 percent per hour) (*15*).

When no cycloheximide was added to the medium, tyrosine production from cell protein still occurred but at a 30 percent lower rate. Thus, during incubation, reticulocytes were in a state of net protein degradation; that is, new protein was synthesized at about one-third the rate of protein degradation. Net protein breakdown in reticulocytes appears reasonable since their maturation into erythrocytes results in a net decrease in cell mass and density (*1*). Supplementing the medium with plasma concentrations of amino acids or insulin did not reduce proteolysis in reticulocytes (Table 1), although it does so in liver and muscle (*14, 15*).

The amount of tyrosine released per milliliter of red blood cells appeared to be directly proportional to the number of reticulocytes in the cell suspension. Thus, the rate of protein degradation was linearly related to the percentage of reticulocytes present in each preparation (Fig. 2), and erythrocyte preparations (< 2 percent reticulocytes) had a very low level of proteolysis (Fig. 1A). Either mature erythrocytes have lost the degradative system, or they contain it but lack appropriate substrates. We have found that erythrocytes do contain a latent proteolytic system that degrades cell proteins when they are damaged (*16*). Although exposure of red blood cells to phenylhydrazine oxidizes cell proteins and promotes their degradation (*16*), the proteolysis observed in reticulocytes (Fig. 2) is not caused by this agent, since if phenylhydrazine were present, proteolysis would also have been seen in erythrocytes (*16*). In addition, when reticulocytosis was maintained by bleeding the animals daily for up to 10 days after the last phenylhydrazine injection, protein degradation could still be demonstrated in the reticulocytes, and the rate of tyrosine production was indistinguishable from that in reticulocytes obtained only 2 days after the last phenylhydrazine injection (Fig. 2). These results and studies with animals never exposed to phenylhydrazine (*17*) indicate that net protein breakdown is an inherent property of the reticulocytes.

To determine whether this process was catalyzed by the soluble ATP-dependent system or by a lysosomal route, we examined the effects of inhibitors of these pathways. When cells were depleted of intracellular ATP by exposure to an uncoupler of oxidative phosphorylation (2,4-dinitrophenol) and to an inhibitor of glycolysis (2-deoxyglucose), tyrosine production from protein decreased by 75 percent (Table 1 and Fig. 1B). These agents similarly inhibit the degradation of abnormal proteins containing amino acid analogs in these cells (*4–6*). Proteolysis was also reduced by *N*-ethylmaleimide and *o*-phenanthroline, which at these concentrations inhibit the rapid breakdown of analog-containing polypeptides in reticulocytes and reticulocyte lysates (*5*). Hemin decreases the degradation of newly synthesized proteins in reticulocytes by inhibiting the soluble ATP-dependent system (*18*). Treatment of reticulocytes with hemin also reduced the degradation of endogenous cell proteins (Table 1), and intracellular hemin may be a physiological regu-

Table 1. Effects of inhibitors on protein degradation in reticulocytes. These data are representative of at least three experiments. In an experiment, duplicate incubations agreed within 5 percent.

Addition to medium	Tyrosine release (nmole/ml-hour)	Inhibition (%)
Experiment 1		
None	50.6	
2,4-Dinitrophenol (0.2 m*M*) + 2-deoxyglucose (20 m*M*)	12.8	75
Plasma amino acids (0.5 m*M*)	48.6	4
Insulin (0.1 unit/ml)	52.5	0
Experiment 2		
None	55.8	
N-Ethylmaleimide (5 m*M*)	9.0	84
o-Phenanthroline (1 m*M*)	33.3	40
Hemin (500 μ*M*)	40.5	27
Experiment 3		
None	54.6	
Chloroquine (10 μ*M*)	49.7	9
Leupeptin (30 μ*M*)	50.1	8

lator of proteolysis in reticulocytes (*18*).

In contrast, proteolysis was not diminished by addition of leupeptin or chloroquine (Table 1), which, in other mammalian cells, reduce overall protein breakdown by effects on the lysosome. Leupeptin inhibits several lysosomal proteases (*19*) and perhaps the soluble Ca^{2+}-activated protease (*20*), whereas chloroquine accumulates within the lysosome where it raises the *p*H and thereby decreases proteolysis (*21*). Ammonia, which acts like chloroquine on intralysosomal proteolysis (*9*), did not affect tyrosine production in the reticulocytes. None of these agents affect the ATP-dependent pathway that degrades abnormal proteins.

These findings indicate that the degradation of reticulocyte proteins associated with cell maturation is not a lysosomal process and is catalyzed by the soluble ATP-dependent pathway. The involvement of this pathway was further tested by experiments with cell lysates. Production of tyrosine from reticulocyte proteins was also observed in soluble extracts (*p*H 7.8) of reticulocytes prepared as in (*5*). This process was almost completely dependent on ATP (Table 2) and was not detectable in extracts of erythrocytes, in accord with the results on intact cells (Fig. 1 and Table 1). Furthermore, proteolysis in the lysates was inhibited by *N*-ethylmaleimide, *o*-phenanthroline, and hemin, as observed with intact reticulocytes (Table 2). Thus, the inhibition in intact cells probably involves direct effects on the nonlysosomal ATP-dependent pathway. In related studies, we have purified from reticulocytes and erythrocytes (*22, 23*), an ATP-stimulated endoprotease that appears to be the rate-limiting protease in this pathway. The only other alkaline protease in these lysates that can degrade aberrant globins is the Ca^{2+}-activated protease (*20*). However, its requirement for Ca^{2+} and inhibitor sensitivity indicate that it is not involved in the soluble ATP-dependent pathway (*22, 23*).

Thus, in addition to catalyzing the breakdown of abnormal proteins, this pathway is responsible for the programmed degradation of proteins during reticulocyte development. The mechanism by which some reticulocyte proteins become substrates for degradation during cell maturation is an unanswered question. They may be specifically modified [for example, by lipoxygenase (*17*)] and then recognized as abnormal polypeptides by the ATP-dependent degradative pathway. Alternatively, these proteins (unlike those also present in erythrocytes) may be inherently unstable and

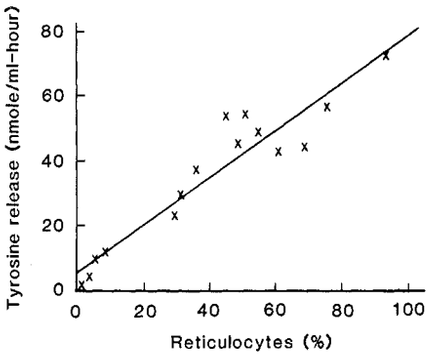

Fig. 2. Relation between the rate of protein degradation and the fraction of reticulocytes in a red cell preparation. Cells were drawn from rabbits (*5*) on the third to tenth day after the last phenylhydrazine injection. Each point represents the average tyrosine production in two flasks incubated in parallel, which agreed within 5 percent. The line was determined by linear regression analysis (r = .95).

may denature and then be selectively degraded by this pathway. This process would prevent the accumulation of inactive, partially denatured proteins in the cell, and the disappearance of these components during cell maturation would result from their hydrolysis in the absence of continued synthesis.

Since these studies were completed, Muller *et al.* (*17*), using an isotopic dilution method, also demonstrated net protein breakdown during reticulocyte maturation. They suggest that the proteins being hydrolyzed are mainly of mitochondrial origin, although our data indicate degradation of soluble (Table 2) as well as particulate proteins. Our simple and highly sensitive method should be useful in studies of reticulocyte maturation in normal and disease states and in studies of the degradation of abnormal human hemoglobins, such as in the unstable hemoglobinopathies and thalassemia (*15, 24*).

Table 2. Degradation of endogenous proteins in reticulocyte extracts. Reticulocyte lysates were prepared and dialyzed as in (*5*) but were centrifuged at 40,000*g* for 1 hour. Samples of lysate containing 0.5 m*M* cycloheximide were incubated at 37°C for 1 hour. At the start of the incubation and after 1 hour, samples (0.5 ml) were removed, and the concentration of acid-soluble tyrosine was determined. When similar preparations were obtained from erythrocytes, no significant proteolysis was observed.

Inhibitor	5 m*M* ATP	Tyrosine production (nmole/hour)	Inhibition (%)
None	+	9.41	0
None	−	0.18	99
N-Ethylmaleimide (5 m*M*)	+	0.56	95
o-Phenanthroline (1 m*M*)	+	6.02	36
Hemin (500 μ*M*)	+	0.56	95

FRANCEE S. BOCHES*
ALFRED L. GOLDBERG
Harvard Medical School, Department of Physiology, Boston, Massachusetts 02115

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* Present address: Clinical Assays, 600 Memorial Drive, Cambridge, Mass. 02139.

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