Life Span of Reticulocytes in Paroxysmal Nocturnal Hemoglobinuria

By S. Y. KAN AND FRANK H. GARDNER

Previous observations have demonstrated the shortened life span of the red cell in patients with paroxysmal nocturnal hemoglobinuria (PNH).1,2 Studies of the survival of homologous PNH red cells by the Ashby technic and of autologous PNH cells labeled with radioactive sodium chromate have demonstrated a short and biphasic red cell survival curve in the patient and normal recipient. This life span pattern has suggested that at least two cell populations exist. Recent observations have also indicated that erythrocyte acetylcholinesterase (ACh) is low in this disorder. The reticulocyte has a lower concentration of ACh and is more susceptible to hemolysis in acidified serum.3

This present study was designed to study the separate in vivo survival of young (reticulocyte-rich) and old (reticulocyte-poor) red cells in PNH patients. The survival of reticulocytes from patients with pernicious anemia during the onset of remission was studied in a similar fashion. These comparisons provide data which suggest that the red cell lesion in PNH is heterogeneously distributed.

Materials and Methods

Two male patients with PNH were studied. M. P. has had the diagnosis for 18 years and S. P. for the past 4 years. The diagnosis was established by the following criteria: hemolytic anemia, positive Ham test indicating excess hemolysis in acidified serum, positive Crosby test, decreased cholinesterase activity of the red blood cells, hemosiderinuria, and hemoglobinemia.

Separation of red cells by differential centrifugation was performed at least 90 days after transfusion therapy so that the patient had a minimal concentration of homologous normal red cells. The patients had hematocrits ranging between 17 per cent to 23 per cent and reticulocyte counts from 15 per cent to 28 per cent at the time of the studies.

Three patients with classical pernicious anemia were studied during the reticulocyte response following cyanocobalamin therapy.

Separation and Labeling of Red Cells According to Density

The method of separation was based on the previous observations that demonstrated a good correlation of red cell age with density.4,5 One hundred and sixty ml. of blood were collected in 20 ml. sterile heparinized, vacuum-sealed test tubes* and centrifuged at 800 rpm (125 x g.) for 15 minutes. The plasma was aspirated in sterile test tubes and...
centrifuged for 10 minutes at 2500 rpm (1000 x g.) to remove platelets and white blood cells. With sterile pipettes, the upper third of the red cell mass was removed from each test tube and pooled in sterile test tubes (A). The middle third of the red cell column was discarded. The lower third was collected with different sterile pipettes and pooled in another set of test tubes (B).

The red cells in tubes A and B were resuspended in the cell free sterile plasma and centrifuged again at 800 rpm (125 x g.) for 15 minutes. The upper and lower third portions respectively of tubes A and B were removed again as described above. The reticulocyte counts were determined, and if the count in the uppermost third of tube A was less than 50 per cent or more than 10 per cent in the lower third of tube B, the procedure was repeated again to obtain these percentages. The final upper or lower third red cell—plasma fractions were incubated with 75 to 150 µc. of NaCr51O4 for 20 minutes at 37 C. Thereafter 100 mg. of ascorbic acid was added prior to injection, and the autologous A or B red cells infused into the PNH patient. Blood samples were drawn frequently for measurement of the whole blood Cr51 activity. Surface scanning of the liver, spleen and heart was performed immediately after injection of the young (reticulocytes) red cells to determine if there was transient localization of these cells in reticuloendothelial organs.6

The acid hemolysis test was performed by a modification described by Gardner and Laforet.7 To determine the maximum number of cells susceptible to acid hemolysis, repeated changes of fresh acidified serum were added to the initial red cell suspension as originally described by Hickey and Malley.8 After each application of fresh serum, the residual percentage of reticulocytes was counted.

The reticulocyte-rich red cells in pernicious anemia were collected and labeled in a similar fashion during the peak of the reticulocyte response on the seventh or eight day following cyanocobalamin therapy. The autologous-labeled young cells were infused to determine if there was transient localization of these cells in reticuloendothelial organs. The acid hemolysis test was performed by a modification described by Gardner and Laforet.7 To determine the maximum number of cells susceptible to acid hemolysis, repeated changes of fresh acidified serum were added to the initial red cell suspension as originally described by Hickey and Malley.8 After each application of fresh serum, the residual percentage of reticulocytes was counted.

The reticulocyte-rich red cells in pernicious anemia were collected and labeled in a similar fashion during the peak of the reticulocyte response on the seventh or eight day following cyanocobalamin therapy. The autologous-labeled young cells were infused to determine the life span of this population in 3 patients. In 1 of these studies the reticulocyte-rich blood was also given to a compatible normal recipient.

RESULTS

In both PNH patients, the estimate of Cr51 red cell life span of the reticulocyte-rich (young) cells was followed by a study of the reticulocyte-poor (old) cells. In figure 1, the life span data are plotted and indicate that the former was significantly shorter than the latter. In the case of M. P., the reticulocyte-poor old cells showed a biphasic curve of survival, whereas the rereticulocyte-rich young cell population had a shorter linear survival curve. The biphasic pattern of the bottom layer is attributed to the young cells present in the separation (reticulocytes 9.1 per cent). In contrast, the study of the old cells in S. P. showed a remarkable linear survival curve which reflects the low reticulocyte concentration (2.9 per cent). The duration of the radioactive red cell survival studies was limited by the time available before transfusions were required. These patients were studied when a maximum reticulocyte count was present to improve the separation of young and old cells. If the life span of the labeled young cells were to be followed for a longer period, a biphasic curve might be observed because reticulocytes comprised only 62 and 67.5 per cent of these samples. Since Cr51 was employed as the label for both populations, the two groups of cells could not be compared simultaneously. However, the hematocrit changes and the amount of hemoglobinuria were nearly identical in both studies so that possible variability in overall hemolytic activity could not account for the dif-
Fig. 1.—The Cr\textsuperscript{51}-labeled red cell survival of reticulocyte-rich (top layer) and the reticulocyte-poor (bottom layer) cells in 2 patients with PNH.

ferences. Both studies were repeated in M. P. 4 months later and the results were similar.

In contrast to the results observed in PNH, labeled reticulocyte (young cells) suspensions in 3 patients treated for pernicious anemia had a Cr\textsuperscript{51} apparent T\textsubscript{1/2} of 50 days in 2 studies. In the third patient the apparent T\textsubscript{1/2} was 67 days (fig. 2). Progressive increments in the red cell volume may have altered the Cr\textsuperscript{51} life span curve during the recovery phase of pernicious anemia. While it is difficult to imagine that a more prolonged life span curve could be obtained, the possibility was discarded in the pernicious anemia life span studies by the following procedures. The reticulocyte-rich upper layer was divided into two portions in patient G. W. One portion was autotransfused to G. W. and the other portion given to a patient, J. S., with polycythemia vera during remission. The life span curves were almost identical.

In another pernicious anemia patient, J. M., in whom a reticulocyte survival was measured, the blood volume was 4570 ml. with 1280 ml. of red cells at the beginning of the life span study. At the end of the study 48 days later, the blood volume was 4580 ml. and the red cell volume was 2150 to emphasize that there was no change in the total circulating volume to influence the life span curve. The pernicious anemia patient F. H., who received a 95 per cent reticulocyte suspension, had a T\textsubscript{1/2} Cr\textsuperscript{51} life span of 62 days (fig. 2).

Red cells prepared in upper and lower fractions were subjected to acid hemolysis at varying pH. The upper fraction consistently showed a more pronounced hemolysis at all pH levels tested (fig. 3). The maximum amount of hemolysis was always obtained at pH 6.4-6.5. In one study, whole blood from M. P. was subjected to repeated acid hemolysis by the addition of fresh acid serum. The reticulocyte count declined with each incubation of fresh acidified serum (fig. 4).
Fig. 2.—The survival of the Cr⁷¹-labeled reticulocyte-rich blood in 3 patients with pernicious anemia during response to therapy. J. S. received reticulocytes prepared from G. W. Reticulocyte percentage is that of the labeled top (reticulocyte rich) cells.

Fig. 3.—Acid hemolysis curve of different fractions of blood prepared from a patient with PNH.

Surface isotope scanning was done immediately after the labeled young red cells (reticulocytes) were infused in the PNH patients as well as J. H. with pernicious anemia. None of the patients showed increased activity (counts) over the liver or spleen to imply that there was transient or permanent sequestration of the labeled reticulocytes.
Fig. 4.—Reticulocyte counts of a sample of whole blood from PNH after repeated hemolysis at pH 6.5 with fresh samples of normal acidified human serum.

Discussion

These studies demonstrate and confirm that the young reticulocyte-rich red cell population is more susceptible to hemolysis in vitro and in vivo than the older, reticulocyte-poor red cell population. These in vitro observations are in agreement with those of Metz and co-workers, though they do not support the previous findings of Dacie and Mollison and Hickey and Malley. Possibly the separation technic with higher percentages of reticulocytes has accentuated these differences more than obtained previously with whole blood. Mahre et al. have described a patient with PNH who exhibited a marked increase in urinary excretion of Fe⁵⁹ during the initial 48 hours after in vivo labeling with radioiron. This rapid excretion could be explained either by a rapid destruction of reticulocytes or by intramedullary cell death. The latter has been described with other hemolytic states. Probably both mechanisms are present in this disorder. The observations reported herein demonstrate quite clearly that the PNH reticulocytes (or young cells) are destroyed more rapidly after they enter the circulation.

The rapid destruction of the reticulocyte in PNH patients appears to be compatible with the accelerated reticulocyte destruction observed in the dog and rodent. However, other than in this disorder such a rapid response
has not been observed in man. Certainly the reticulocytes produced following cyanocobalamin therapy in pernicious anemia are not selectively destroyed. After correction for chromium elution, this almost "pure" cohort of reticulocytes in pernicious anemia had a finite life span with no random destruction. The slightly shorter life span in the other patients with pernicious anemia probably is related to the more inadequate differential separation of young cells. The reticulocytes in response to nutritional therapy in 3 patients with pernicious anemia showed no evidence of rapid destruction or sequestration in the liver or spleen by the technic used.

The selective rapid destruction of reticulocytes has thus appeared to be a phenomenon peculiar to PNH. However, recently another intracorpuscular red cell defect, pyruvate kinase deficiency, was studied in a patient when the reticulocytes were greater than 50 per cent of the peripheral blood. The $T^{1/2}$ in this reticulocyte-rich blood was 3 days, similar to the observations in PNH. These data support the rapid destruction of intracorpuscular defective reticulocytes in another disorder. Further data are needed to determine whether an older cell population has a longer life span in pyruvate kinase deficiency.

It appears certain that of all the reticulocytes produced at one time in PNH a portion must survive longer than others. This may occur in two ways: (1) The reticulocytes may vary in their degree of stromal abnormalities so that the less abnormal red cells survive longer and constitute the bottom (older) fraction of the cells on separation. Dacie has postulated the presence of abnormal clones producing the PNH red cells, and this variability of abnormalities may represent cells produced by different clones. (2) Alternatively, as the red cells mature in the blood stream, a portion may acquire some protective feature, rendering them less susceptible to hemolysis. During in vitro acid serum hemolysis, the red cells that escape lysis have a protein bound to the red cell membrane. This protein coating will cause the red cells to agglutinate with antisera prepared against human 11S globulins (complement). Such an observation suggests that the reticulocytes are hemolyzed during the acid serum test because complement proteins can bind more avidly to the "sticky" membrane. The older cells that escape hemolysis do not bind complement protein strongly enough to initiate immune hemolysis. This speculation has further support in that the increased in vitro hemolysis of PNH reticulocytes implies a membrane defect more responsive to the fluid phase of complement interaction. We may infer that the PNH reticulocyte has more binding sites available for complement component attachment. As the red cell ages the binding sites decrease so that there is less chance for destruction by random attachment of the activated fluid phase of complement. There has been no correlation made in this patients between the life span and reticulocyte percentages. However these speculations regarding in vivo and in vitro observations may aid in understanding hemolytic mechanisms in this disease.

**Summary**

Two patients with PNH were transfused with young and old Cr$^{51}$-labeled red cells. The young red cells (reticulocytes) were destroyed more rapidly...
than the older red cells. In vitro studies also revealed that the reticulocyte was more susceptible to acid hemolysis in acidified serum.

In contrast the Cr\(^{51}\)-labeled reticulocytes in 3 patients with pernicious anemia responding to cyanocobalamin showed a normal life span.

In the patients with PNH and pernicious anemia, no sequestration of Cr\(^{51}\)-labeled reticulocytes was noted in the liver or spleen by surface scanning.

**SUMMARIO IN INTERLINGUA**

Duo patientes con paroxysmic hemoglobinuria nocturne recipivava transfusiones de juvene e vetule erythrocytos marcate con Cr\(^{51}\). Le juvene erythrocytos (reticulocytos) esseva destruite plus rapidemente que le erythrocytos plus matur. Studios in vitro revelava etiam que le reticulocytos esseva plus sensibile pro hemolyse acidic in sero acidificate.

Per contrasto con iso, reticulocytos a Cr\(^{51}\) monstrava un longevitate normal in 3 patientes con anemia perniciosse que respondeva a cyanocobalamina.

In le patientes con paroxysmic hemoglobinuria nocturne e anemia perniciosse, nulle sequestration de reticulocytos marcate con Cr\(^{51}\) esseva notate in le hepate o in le splen per scrutinage al superficie.

**REFERENCES**


S. Y. Kan, M.D., Clinical Fellow in Medicine at the Royal Victoria Hospital, and Research Fellow, Medical Research Council of Canada; Former Trainee in Hematology U. S. P. H. S., Peter Bent Brigham Hospital and Harvard Medical School, Boston, Mass.

F. H. Gardner, M.D., Associate Clinical Professor of Medicine, Harvard Medical School; Physician, Peter Bent Brigham Hospital, Boston, Mass.
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