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Circadian Variation in Reticulocyte Counts and Immuno-detectable Erythropoietin Titers[1] (37997)

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(Introduced by R. M. Des Prez)

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In recent years studies have revealed that many body and organ functions are not constant but fluctuate within a 24-hr period. Such fluctuations include the concentration of plasma iron (1–4), the mitotic index of the bone marrow (5), and the urinary erythropoietin concentration (6). Investigations of the blood reticulocyte percentage have produced conflicting results. Some studies have shown that the blood reticulocyte percentage varied throughout the day (7), while others did not demonstrate such a variation (8). No studies have been performed on plasma erythropoietin levels since bioassay procedures are too insensitive to detect the minor variations in the normal levels (9).

In the present study reticulocyte counts and immunodetectable plasma erythropoietin levels were measured throughout the day in normal subjects to determine if a fluctuation was present. These measurements were compared with the fluctuations in plasma iron concentration that occurred in these same subjects.

Methods. Studies were performed on nine normal subjects. In normal subjects 1–4 erythropoietin levels alone were measured while in subject 9 only reticulocyte counts were performed. In subjects 5–8 both reticulocyte counts and erythropoietin titers were performed. All subjects studied were normal males between 25 and 40 yr of age with normal hemotocrit values throughout the study. In all these subjects at least five specimens were obtained each day. Reticulocyte counts were performed on blood stained with new methylene blue, fixed and counterstained with Wright's stain. This allowed smears to be saved and counted at leisure (10). The reticulocyte count reported is the result of counts performed by two people on 2000 red blood cells each for a total of 4000 red blood cells for each sample point. The same two individuals counted all slides independently of each other.

Erythropoietin levels in the blood were determined on serum samples using an immunoassay previously reported (11). Specimens were run in duplicate at two separate starting dilutions to allow a better opportunity to detect for variations in levels (12). The reproducibility of the hemagglutination-inhibition technique has been tested by determining the units of erythropoietin in the serum of four patients and a normal subject each month for 4 mo (13). The results showed excellent agreement between the monthly determinations. Only one value of the 20 determinations was more than one doubling dilution higher than the initial value. In another study, 36 different sera were assayed in triplicate. The sera were then given to another investigator who divided each specimen into two portions and gave them code numbers. The amount of erythropoietin was redetermined in triplicate for each coded specimen. In 103 of the 108 determinations, the results were

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either the same or only one doubling dilution different. To test the precision and sensitivity of the assay, and as a further test of reliability, 10 independent determinations of the amount of erythropoietin in a single sample were made by this technique. The values in these 10 determinations varied from 0.82×10^{-2} to 1.95×10^{-2} units of erythropoietin. However, nine of the determinations were between 0.82×10^{-2} and 1.15×10^{-2} with a mean value of $1.1 \pm 0.2 \times 10^{-2}$.

In the present study no change in concentration was considered significant unless the concentration changed by a factor of 2 (two tube dilutions). The serum iron was determined on these specimens using the method of Ramsay (14).

Results. Reticulocyte counts. Reticulocyte counts were performed on subjects 5–9. Counts were obtained for 3 consecutive days in subjects 5, 6, and 8 while they were obtained for 2 consecutive days in subject 7 and 4 days in subject 9. In every subject (Fig. 1) there was variation in the reticulocyte count throughout the day. The reticulocyte counts varied from the lowest value to highest value by at least a factor of 2 on all 15 subject days. To determine if these counts were varying significantly, all reticulocyte counts for a subject were averaged and the standard deviation determined for that count (10). There were 100 individual reticulocyte counts and 35 of these fell outside the values expected for 2 standard deviations. Subject 7 had the least number of reticulocyte counts beyond 2 standard deviations of the mean value (4 of 16) while subjects 5 and 6 had the most (10 of 18 in each).

The times that the reticulocyte counts were maximal were consistent in subjects 5, 6, and 9 (8 PM, 8 PM, and 2 PM) but in subjects 7 and 8 the maximal value was seen at various times from day to day. In 15 of the study days, the peak reticulocyte counts were between 2 PM and 8 PM, though one subject had a second peak about 2 AM on one occasion (subject 9) (Fig. 1).

Erythropoietin levels. Erythropoietin levels were measured with the immunological technique in eight subjects. Figure 2 illustrates the results seen in these subjects. In subjects 5, 6, and 8 levels were measured consecutively for 3 days while in subjects 1–4, and 7, values were measured for 2 days. A variation in the daily erythropoietin level was detected in every subject except subject 5 (Fig. 2). Of the 24 test days available, a variation in titers of greater than 2 was noted on 17 occasions. On two other occasions the increase was borderline. On five occasions the change in concentration was less than a factor of 2. The levels of erythropoietin varied greatly. Values over 100 milliunits per ml were recorded on five of the 21 subject days (Fig. 2). Low values for the day usually were 30 mU per day or less. Usually maximal concentration occurred in the afternoon or evening (14 of the 17 with definite peaks). In the same individuals the time the maximal value occurred varied from day to day. Comparison of individual subject values indicated that

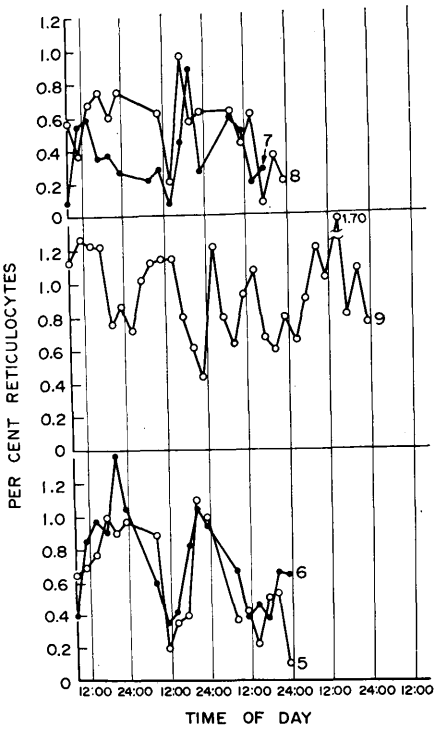

FIG. 1. Reticulocyte counts in five normal subjects. The percentage was determined from counts on 4000 red blood cells at each point.

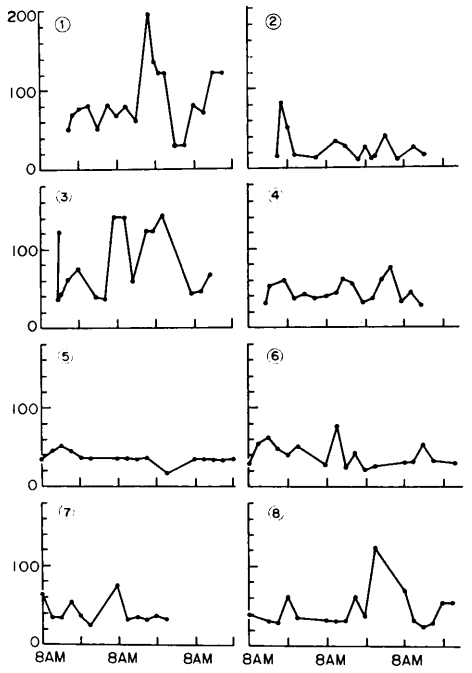

FIG. 2. Erythropoietin levels in milliunits per milliliter of serum. For changes in levels to be significant the concentration must change by a factor of 2.

the maximal erythropoietin level and maximal reticulocyte count often occurred some hours apart, though on the average both tended to occur at approximately the same time. Likewise, serum iron concentration failed to correlate in time with reticulocyte and erythropoietin values as maximal values for serum iron occurred in the morning hours soon after the subjects arose from sleep.

Discussion. Function of many organs seems to fluctuate during the day in an orderly manner. Often these fluctuations are difficult to detect because the sensitivity of the assay techniques is limited and does not allow the detection of minimal variations. Reticulocyte counts are simple and easy to do, but certain pitfalls in the technique limit the significance of the results. First, the reticulocyte count may vary due to random variation of their distribution on the slide being counted (10). The more red blood cells that are counted, the less the chance that random variation will play a part. Thus, with a reticulocyte count of 1%, the true value might vary from 0.4% to 1.6%, with the traditional method of counting 1000 red blood cells (two standard deviations) (10). By counting 4000 red blood cells, the range of values for a 1% value would be 0.7%–1.30% (10). To reduce this error of distribution further, a count on 10,000 red blood cells or more would be necessary. Such counts would be very time consuming and probably have an increased error due to fatigue of the personnel doing reticulocyte counts. To reduce fatigue, only 2000 red blood cells were counted by the individuals performing the present study and all reticulocyte counts were performed on permanent slides thus allowing the determinations to be made at a time when fatigue was not present. Also, variations in reticulocyte counts can occur due to errors in recognition of reticulocytes by laboratory personnel. To reduce this error, the same two individuals performed all counts. Rarely did differences between their counts appear significant and counting an extra 2000 cells did not change the percentage of reticulocytes significantly on any occasion. Thus, by counting 4000 red blood cells on permanent reticulocyte slides and having the same two people perform the counts, the errors of the technique were reduced as much as possible.

In a similar manner, the erythropoietin assay used in these experiments has limitations. Animal assays are too insensitive to detect normal plasma levels of erythropoietin (9). The immunoassay is performed by serial dilutions of test material reacted with a constant amount of antiserum to erythropoietin and red blood cells sensitized with an erythropoietin preparation as the indicator for the reaction. A standard is run with each preparation so that the results can be expressed in units of erythropoietin. To date, this assay appears specific for erythropoietin (11, 12, 13). Two different starting dilutions of test serum were run with this assay to increase the sensitivity of the assay and each dilution was assayed in duplicate. However, this still requires that the estimated concentration change by a factor of 2 to be significant. Thus, large changes in concen-

tration are necessary before a change can be considered significant.

In all subjects, there was a constant change in the percentage of reticulocytes present in a 24-hr period. These changes were significantly greater than would be accounted for by variation in random distribution of the reticulocyte counts. These studies confirm that there is a variation in reticulocytes in man similar to that recently reported in animals (7). It is difficult to say that the rhythm of variation is one of exactly 24 hr but it is close to this. It would take many more days of reticulocyte counts to obtain enough points for accurate determination of this time. Of note is that in several subjects reticulocyte counts usually appeared to be lower on the third day of study than on the first day. Thus, it may not be easy to determine the exact time cycle since the effect of fatigue and abnormal sleep patterns induced by testing may interfere and cause a disruption of the cycle before enough data can be obtained.

There also appears to be a variation in erythropoietin values determined by immunoassay procedure. It is difficult to define this cycle exactly since many more days of data would be required and an assay which is even more accurate than the present immunotechnique. Thus, our data reveal that daily variation in reticulocyte count and erythropoietin level appear to exist in normal subjects.

Summary. Immunoreactive erythropoietin levels of serum and reticulocyte counts were measured in a group of normal subjects. Values fluctuated throughout the day. Maximal levels of reticulocyte counts and erythropoietin tended to occur in the afternoon and early evening but the times did not coincide in individual subjects.

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