# The Periodic Acid-Schiff Reaction in Neutrophil Leukocytes in Untreated and Myleran-Treated Chronic Myelocytic Leukemia

## A Quantitative Microspectrophotometric Study

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A REDUCTION IN THE MEAN AMOUNT of periodic acid-Schiff reactive material (PASMa) in neutrophil leukocytes from patients with chronic myelocytic leukemia (CML) has previously been noted with semiquantitative "score" methods.[1-3] The PASMa studied in these investigations proved to be digestible with saliva or diastase and was therefore considered to consist of glycogen. So far, no attempt has been made to follow a possible quantitative change in the reaction at various stages of the leukemia during treatment. This is probably due to the obvious difficulty of obtaining accurate results with the "score" methods due to the rather intensive PAS reaction of both normal and leukemic neutrophil leukocytes. Recently, a microspectrophotometric method has been developed for the quantification of the PAS reaction in single cells,[4,5] thus permitting accurate studies of both the mean amount of PASMa in cells and the frequency distribution of PASMa per cell. In the present work, the quantitative method has been applied to an investigation of the PAS reaction in neutrophil leukocytes from patients with CML before and during treatment with Myleran.

## MATERIAL AND METHODS

### *Material*

The material was obtained from Tufts Hematology Laboratories, Boston City Hospital, Boston, Mass., the Children's Cancer Research Foundation, Boston, Mass., the Department of Medicine and the Department of Radiology, University Hospital, Lund, Sweden, and the Department of Medicine and the Radiumhemmet, Karolinska Sjukhuset, Stockholm, Sweden.

The control series consisted of 20 normal healthy persons (Table 1). The pathological series (Table 1) consisted of 23 cases of CML, divided into three groups, namely CML 1 (untreated or in relapse), CML 2 (in partial remission), and CML 3 (in remission). Eight cases of CML were studied separately in a special investigation (Table 3).

The CML 1 group consisted of 10 cases. Six of these cases were newly discovered and had not been treated previously. Four of the cases, treated 3–36 months earlier with Myleran, were in relapse. WBC for the entire group was 44,000–555,000 (arithmetic

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**Table 1.—*Clinical Material***

| Diagnostic Group | Number of Cases | Age Mean | WBC × 1000 Mean | Immature Cells in Peripheral Blood Per Cent Mean | Number of Neutrophils Measured: Analysis of variance | Number of Neutrophils Measured: Special studies |
|---|---|---|---|---|---|---|
| Normal healthy persons | 20 | 29 | | | 1000 | 200 |
| CML 1 | | | | | | |
| Newly discovered, never treated | 6 | | | | | |
| Relapse | 4 | | | | | |
| Total | 10 | 50 | 144 | 35 | 500 | 500 |
| CML 2 | | | | | | |
| Partial remission | 5 | 46 | 14 | 10 | 250 | 50 |
| CML 3 | | | | | | |
| Remission | 8 | 44 | 10 | 3 | 400 | 200 |
| *Total* | 43 | | | | 2150 | 950 |

**Table 2.—*Group Mean and Estimated Variance for the Total Extinction at 546 mμ in Single Neutrophils from Normal Persons and Patients with Chronic Myelocytic Leukemia (see text)***

| Diagnostic Group | Number of Subjects | Number of Cells | Group Mean | Confidence Interval 99 Per Cent for Group Mean | Estimated Variance: Between subjects | Estimated Variance: Between cells within subjects |
|---|---|---|---|---|---|---|
| Normal series | 20 | 1000 | 102 | ±8 | 151 | 219 |
| CML 1 (untreated or in relapse) | 10 | 500 | 59 | ±12 | 123 | 166 |
| CML 2 (partial remission) | 5 | 250 | 77 | ±20 | 91 | 266 |
| CML 3 (remission) | 8 | 400 | 101 | ±11 | 76 | 375 |

mean 144,000). The differential count in blood and bone marrow was consistent with the diagnosis CML. Cytogenetic analysis of bone marrow smears in 4 cases showed that the Ph[1] chromosome was always present. The alkaline phosphatase reaction in neutrophils was weak or absent in all cases.

The CML 2 group consisted of 5 cases which were under treatment with Myleran but were not in satisfactory remission. WBC was 7000–21,000 (arithmetic mean 14,000). The immature cells in the peripheral blood were 7–15 per cent (arithmetic mean 10 per cent). The diagnosis was made, using the same criteria as for CML 1, before starting treatment. Cytogenetic analysis in 3 cases showed the presence of the Ph[1] chromosome.

The CML 3 group consisted of 8 cases in remission after treatment with Myleran. WBC was 4000–16,000 (arithmetic mean 10,000). The peripheral blood usually contained a few immature myeloid cells (arithmetic mean 3 per cent). The diagnosis was made before treatment using the same criteria as for the CML 1 group. Cytogenetic analysis was made before or during treatment in 6 cases and the Ph[1] chromosome was always present.

## *Methods*

*Sampling and Preparation of Smears.* About 10 ml. of blood was obtained by venous puncture and transferred to an evacuated test tube containing 12 mg. EDTA (Vacutainer B.-D., Columbus, Nebraska). (Heparin was used as the anticoagulant in part of the

**Table 3.—*Chronic Myelocytic Leukemia, Repeated Studies in the Same Patient***

| Patient | Untreated or in Relapse | Partial Remission | Remission |
|---|---|---|---|
| R. P. 1st day | 46 ± 1.2* | | |
| R. P. 7th day | 54 ± 2.5 | | |
| E. L. | 75 ± 1.6 ——→ | 85 ± 1.5 | |
| D. L. | 66 ± 2.1 ——→ | 68 ± 2.2 | |
| P. J. | 76 ± 1.5 ——→ | | 119 ± 2.6 |
| J. D. | 87 ± 2.5 ——→ | | 97 ± 2.4 |
| E. P. | 74 ± 1.7 ——→ | | 94 ± 2.0 |
| E. B. | | 89 ± 3.0 ←—— | 116 ± 1.9 |
| W. B. 1st day | | | 99 ± 1.6 |
| W. B. 63rd day | | | 93 ± 1.3 |

*Standard error of the mean.

Table 3. Repeated estimates of the mean total extinction at 546 mμ ($\overline{E}_{tot}NL$) of neutrophils from individual cases of CML in the same or in different clinical conditions because of treatment. Right arrow (——→) indicates regress of symptoms because of Myleran treatment. Left arrow (←——) indicates progress of symptoms.

material, but this does not affect the results.) The blood was then mixed with 5 ml. of a 1 per cent isotonic human fibrinogen solution (KABI AB, Stockholm, Sweden). The test tube containing the mixture was placed at an angle of 60° to the horizontal plane, at room temperature (20 C.) for 20 to 30 minutes for the red blood cells to settle. About 0.5 ml. of the "white blood cell" part of the supernatant was gently removed with a Pasteur pipette, and smears were made on hemocytometer cover glasses (Bürker). The smears were air dried, fixed in absolute methanol for 15 minutes, and air dried again.

*PAS Staining.* The McManus PAS reaction[6-9] was performed with modifications[4,5]: 0.5 per cent periodic acid for 60 minutes; rinsed in distilled water for 3 minutes (1 minute in each of 3 rinses); Schiff reagent (Fuchsin-Sulphurous Acid Test Solution, Harleco, Hartmann-Leddon Company, Philadelphia, Penn.) for 60 minutes; sulphuric acid rinse (10 per cent sodium metabisulphite 6 ml., normal hydrochloric acid 5 ml., distilled water 100 ml.) for 6 minutes (2 minutes in each of three changes); rinsed in running tap water for 5 minutes; rinsed in distilled water for 30 seconds. The smears were air dried and mounted in immersion oil (Shillaber's n = 1.5150). Control smears were pretreated with α-amylase (α-amylase from hog pancreas, Type 1, aqueous crystalline suspension, 32 mg./ml., Sigma Chemical Company, St. Louis, Missouri) diluted to 1 mg./ml. distilled water.

*Microspectrophotometry.* Absorption measurements on stained neutrophils for the quantitative estimation of total extinction ($E_{tot}NL$) were made at 546 mμ[4,5] in a rapid scanning microspectrophotometer described eleswhere.[10-12] The extinction values were automatically integrated over the neutrophil area and registered for each neutrophil as the total extinction. This was taken as a measure of the PASMa in the cell. All values were related to a normal standardized control,[5] stained and measured at the same time as the pathological specimen. The values were expressed as percentages of the mean value for the control and designated $E_{tot}NL$, relative values. Fifty neutrophils were measured in each case and the mean total extinction $E_{tot}NL$ (subject mean) was calculated. The mean of the subject means was calculated for each group and termed the group mean. Another 25–50 cells were measured, in some cases, to clarify the shape of the frequency distribution of $E_{tot}NL$ per cell. In order to simplify the statistical treatment, these additional measurements were not included in the analysis of variance (see below).

Neutrophils pretreated with α-amylase had $E_{tot}NL$ values of less than 10 per cent of the subject mean for untreated cells.

Absorption spectra from individual PAS stained neutrophils were recorded for qualitative

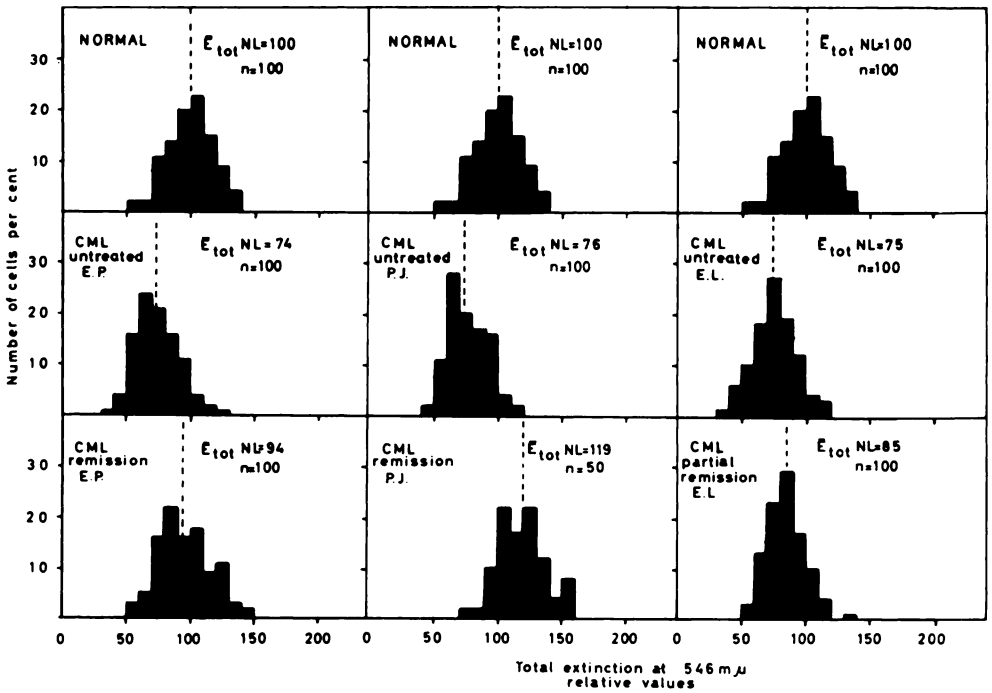


**Fig. 1.—Frequency distribution of the total extinction** at 546 mμ ($E_{tot}NL$) per mature neutrophil leukocyte after the modified PAS reaction in 3 cases of CML before and after treatment with Myleran. Broken line = mean total extinction ($\overline{E}_{tot}NL$ = subject mean).

analysis in a universal microspectrophotometer.[13] No difference in the shape of the curves or in the absorption maxima was obtained in cells from different groups, including the normal one. PAS absorption spectra from normal neutrophil leukocytes have been presented elsewhere.[4]

*Statistical Methods.* Calculations of the standard error of the mean, the confidence intervals, and the analysis of variance were based on conventional statistical methods.[14] The correlation between the number of nuclear lobes and the extinction values was studied for each diagnostic group by factorial arrangement and the data treated as disproportionate sub-class numbers.[15] Levels of significance are denoted:

| | |
|---|---|
| not significant | $p > 0.05$ |
| almost significant | $0.05 > p > 0.01$ |
| significant | $0.01 > p > 0.001$ |
| highly significant | $0.001 > p$ |

## Results

*Normal Series.* The group mean for PASMa (Table 2) was approximately the same as the subject mean for the control to which all values were related.[5] The frequency distribution of PASMa per cell was normal (Fig. 1). The lowest individual value was 49 per cent of the normal group mean and belonged to a case with a subject mean of 97. The highest value was 192 and belonged to a case with the highest subject mean, 129. There was no correlation between the number of nuclear lobes and the extinction values.

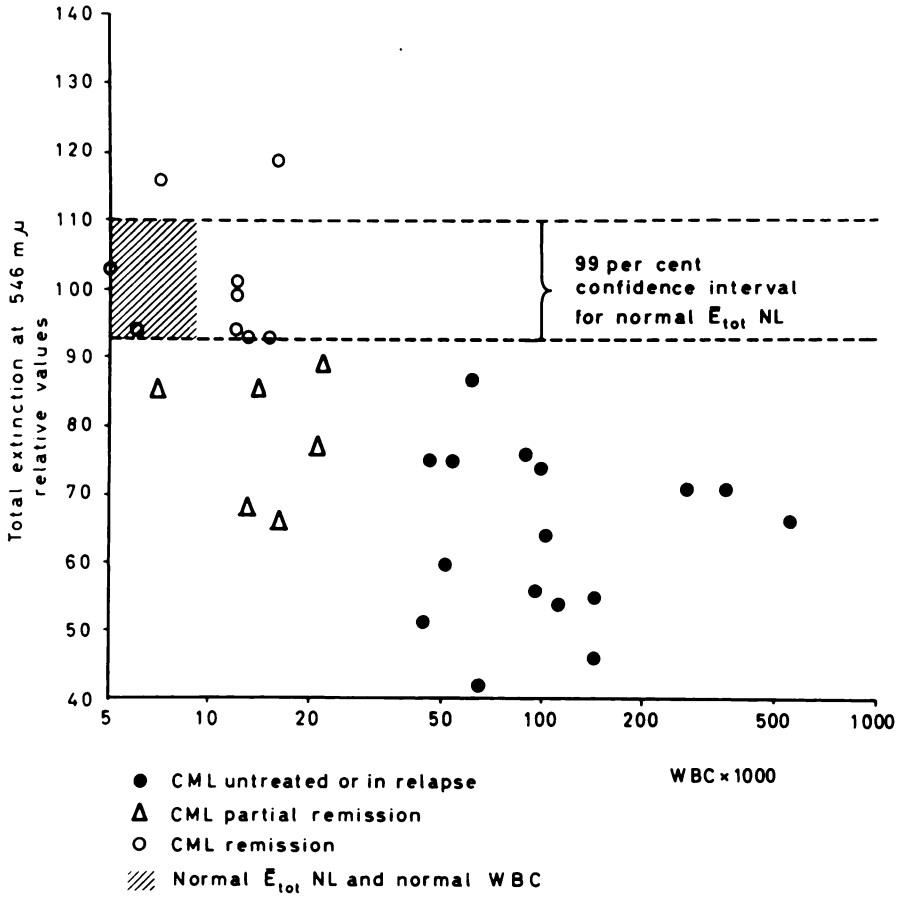


**Fig. 2.—Correlation between the mean total extinction** at 546 mμ ($\bar{E}_{tot}NL$ = subject mean) and WBC in individual cases during the course of Myleran treatment of CML. The figure includes all estimates of $\overline{\bar{E}_{tot}}NL$ in the material.

*CML 1 (Untreated or in Relapse).* The group mean for PASMa in mature neutrophils was only 59 per cent of the control value (Table 2) and differed from all other group means to a highly significant or a significant extent. The frequency distribution of PASMa per cell was normal (Fig. 1). The variance between cells within subjects was somewhat lower than normal. The decrease in PASMa was equally pronounced in cells with different numbers of nuclear lobes and thus could not be ascribed to the relatively increased number of band forms.

*CML 2 (in Partial Remission).* The PASMa showed a tendency to normalization in these cases (Tables 2 and 3, Figs. 1 and 2). The group mean was intermediate between the group means for CML 1 and CML 3 (see below), being larger than the former in a significant extent and smaller than the latter in a highly significant extent. In a special study of 2 cases (Table 3, cases E. L. and D. L.) the same patient was investigated when untreated and in partial remission. One case (E. L.) showed an elevation of PASMa

in partial remission compared to the value before treatment, while the other (D. L.) did not. The first case (E. L.) was close to remission, subjectively well, with almost no anemia, and with normal WBC. The signs of incomplete remission were 8 per cent immature cells in the peripheral blood and palpable spleen and liver. The second case (D. L.), with an original WBC of 555,000, massive splenomegaly, anemia and 29 per cent immature cells in the peripheral blood, was in partial remission after 1 month of treatment with Myleran. There was then a WBC of 13,000 and 10 per cent immature cells in the peripheral blood. She was, however, still in poor condition, anemic and with a palpable spleen. The PASMa did not normalize. As shown in Figure 3, the change from subnormal to normal PASMa usually took place in partial remission, at WBC between 10,000 and 20,000. However, this change was not necessarily a function of the WBC, and the PASMa could remain low if signs of remission other than reduction in WBC failed to appear as in the case D. L. The variance between cells within subjects was close to normal.

*CML 3 (Remission).* The PASMa was completely normalized in remission (Table 2, Figs. 1 an 2). The group mean was practically the same as in the normal series and different from that of CML 1 to a highly significant extent. Special studies of the same patient, untreated and in remission (Table 3, cases P. J., J. D. and E. P.), showed a complete normalization of the PASMa after Myleran treatment. The variance between cells within subjects was somewhat larger than in CML 1.

## Discussion

The PASMa in neutrophil leukocytes probably consists mainly of glycogen,[1-3,16-24] although there has been some objection to this interpretation.[25] Recent investigations of the kinetics of the reaction in neutrophils compared to the kinetics in model systems,[27] as well as quantitative estimation of the influence of $\alpha$-amylase on the reaction in neutrophils,[26] strongly suggest that this reaction depends mainly on the presence of glycogen. It has also been shown that this glycogen is well preserved by methanol fixation and that it is quantitatively stained by the PAS reagents.[26,27] It is thus assumed that the PAS reaction in the neutrophils serves as an approximate measure of the amount of glycogen in the cell. The low values for the PASMa found in untreated CML were consistent with results obtained with semiquantitative "score" methods after PAS staining.[1-3] They also agreed with the finding that the amount of chemically estimated glycogen in separated myeloid cells from CML was 25–50 per cent below normal.[28-30] The reason for this reduction was obscure. It could not be ascribed to the very common augmentation of the number of band forms in CML, because there was no significant difference between the PAS reaction in these cells and in cells with a different number of nuclear lobes. As it has been shown that leukemia neutrophils are deficient in a number of enzymes, it would be convenient to suggest a deficiency in the chain of enzymes responsible for glycogen synthesis. Recent experiments,[29,30] however, have shown a 3-fold to 10-fold increase from normal in the incorporation

of glucose into glycogen in myeloid cells from CML, which suggests that there is no such deficiency in the capacity of the leukocytes to synthesize glycogen. It could be argued that in these experiments the whole growing myeloid cell population of CML was compared to a normal mature neutrophil cell population, which might give a false picture of the glycogen synthesizing capacity of mature neutrophil CML-leukocytes.

The change from low to normal amounts of PASMa after treatment usually occurred in connection with clinical remission after several weeks of treatment. The WBC was then usually around 10,000–20,000. There was no correlation between WBC or duration of treatment and the PAS values before this change to nearly normal WBC. A direct Myleran effect on the mature neutrophil leukocytes could thus be excluded in view of the relatively short lifespan of these cells.[31] It thus seemed more probable that the change to normal values was related to the known Myleran action on precursor cells. The possibility remains that two populations of myeloid cells exist in CML, one predominatingly leukemic and one normal, and that the Myleran had, to some degree, a selective effect on leukemic precursor cells. This would result in a predominately normal neutrophil population after effective treatment. The tendency to normalization of the alkaline phosphatase reaction in neutrophils from the peripheral blood, previously noted in CML in remission[32] supports this hypothesis. The finding that a larger proportion of normal metaphases, lacking the Ph[1] chromosome, might appear in CML after overtreatment compared to untreated CML[33] partly agrees with this view. A preferential release of normal neutrophils from the bone marrow to the blood after treatment might be a complementary possibility. This view needs further clarification.

## Summary

Microspectrophotometric quantification of the periodic acid-Schiff (PAS) reaction has been performed in about 3,000 individual mature neutrophil leukocytes from 20 normal subjects and 23 patients with chronic myelocytic leukemia (CML), of whom 10 were untreated or in relapse and 13 in partial remission or remission.

The PAS reaction was taken as a quantitative measure of the cellular amount of PAS reactive material (PASMa), most probably equivalent to glycogen. The mean amount of PASMa in neutrophils from untreated and relapse cases of CML was 43 per cent less than in normal neutrophils. The frequency distribution of PASMa per cell was normal in individual cases. In clinical remission after Myleran treatment, the neutrophils contained normal amounts of PASMa.

## Summario in Interlingua

Quantification microspectrophotometric del reaction a acido periodic Schiff esseva effectuate pro circa 3000 matur neutrophilos individual veniente ab 20 subjectos normal e ab 23 patientes con chronic leucemia myelocytic, incluse 10 non-tractate o in recidiva e 13 in remission o remission partial.

Le reaction esseva interpretate como indice del quantitate cellular de

material reactive a acido periodic schiff, equivalente probabilemente a glycogeno. Le quantitate medie de tal material in neutrophilos ab subjectos con non-tractate o recidivate chronic leucemia myelocytic esseva inferior per 43 pro cento a illo in neutrophilos normal. Le distribution de frequentia del material per cellula esseva normal in casos individual. In casos in remission clinic post tractamento a Myleran, le neutrophilos contineva quantitates normal de material reactive a acido periodic Schiff.

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