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# Ag-NOR staining and satellite association in bone marrow cells from patients with mycosis fungoides

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Silver staining of nucleolus organizing regions (Ag-NORs) of acrocentric chromosomes and the frequency of satellite association (SA) in bone marrow (BM) cells from 7 patients with mycosis fungoides (MF), were studied. BM samples of 7 normal healthy individuals were taken as controls. The mean number of Ag-NORs per metaphase was increased in patients ($7.20 \pm 0.25$) compared with controls ($5.40 \pm 0.16$) ($p < 0.002$), related with the increase of the D group. Moreover, a significant higher percentage of Ag-NOR positive cells in patients ($71.7 \pm 3.9$) than controls ($48.0 \pm 7.8$) ($p < 0.02$), was seen. The analysis of SA revealed a significant increase in the percentage of cells with 1–2 association pairs (ASPs) in patients with respect to their controls ($p < 0.05$), and a trend to a decrease in the percentage of cells without ASPs. Furthermore, a correlation between the number of Ag-NORs and the mean of ASPs per cell was also found for patients ($r_k = 0.65$; $p < 0.05$). These results may be associated with a certain degree of immaturity, a high proliferative activity and modifications of the growth rate of BM cells in MF patients.

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In normal human cells, the nucleolus organizing regions (NORs) corresponding to the sites of rRNA gene clusters, are localized on the secondary constrictions of the 10 acrocentric chromosomes of D and G groups. Only NORs in which the genes were actively transcribed during the preceding interphase, are stained by silver (MILLER et al. 1976). Frequently, these Ag-NOR chromosomes are involved in associations, giving place to the so-called satellite association (SA) that represents the remnants of the nucleolus. NOR activity varies in different tissues, depending on such factors as cell types and degree of differentiation (MAMAEV and MAMAEVA 1990). Studies in normal bone marrow (BM) cells have shown a lower number and a wider variability of Ag-NORs (REEVES et al. 1982; MAMAEV et al. 1985; ARDEN et al. 1985, 1989), plus a lower degree of SA than in lymphocytes (REEVES et al. 1982).

Studies performed on silver stained metaphases of BM and/or peripheral blood lymphocytes (PBLs) in lymphoid malignancies, have shown heterogeneity in the Ag-NOR pattern. Increased frequencies have been found in BM cells of acute lympho-blastic leukemia patients (ARDEN et al. 1985, 1989). No differences with controls have been observed in cell lines of malignant lymphomas (SCHULZE et al. 1984) and PBLs of patients with mycosis fungoides (PEDRAZZINI and SLAVUTSKY 1991), whereas a significant Ag-NOR increase was observed in PBLs from patients with B-cell chronic lymphocytic leukemia (PEDRAZZINI and SLAVUTSKY 1991).

In this study, we have applied Ag-NOR staining to BM samples from patients with mycosis fungoides (MF), a T-cell chronic lymphoproliferative disorder. Modifications in the NOR activity expressed by the number of Ag-NORs, the percentage of Ag-NOR(+) mitoses, and the frequency of SA were found.

## Materials and methods

BM samples from 7 untreated patients with MF were studied. Simultaneously, BM samples from 7 normal healthy individuals were taken as controls.

BM samples from patients and controls were incubated 24 h at 37°C in F-10 medium with 15 % fetal calf serum. HOWELL and BLACK method

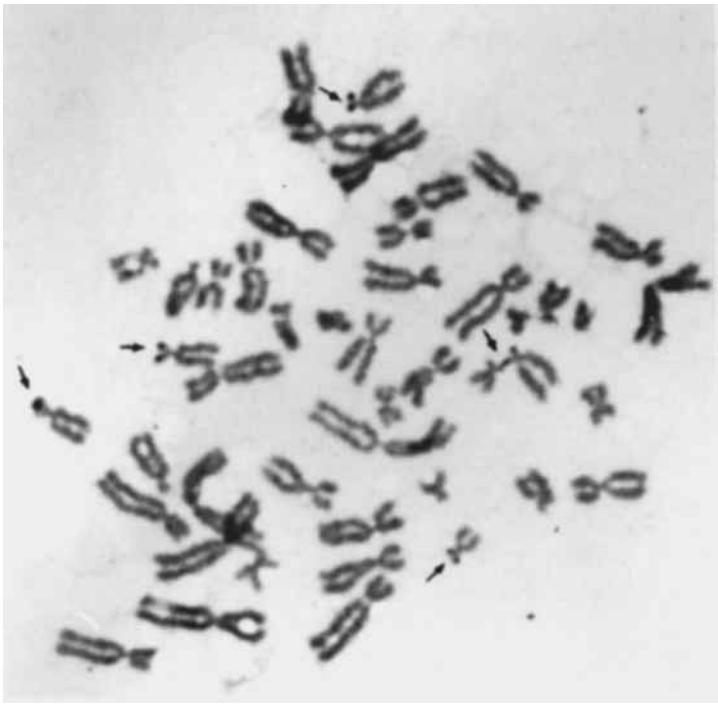

**Fig. 1.** Bone marrow metaphase from a patient with mycosis fungoides showing Ag-NOR staining.

(1980), which uses gelatin as colloidal protector, was employed for Ag-NOR staining. A pretreatment of 10 min in formic acid solution (2N) was made (MAMAEV et al. 1985). A total of 30 metaphases with D and G group chromosomes clearly defined were scored for the mean number of Ag-NORs from each sample. All metaphases which were adjacent to silver-stained interphase nuclei, were analyzed to assess the percentage of Ag-NOR(+) mitoses (Fig. 1).

The degree of SA was studied in the same 30 cells scoring the number of SA complexes by the types of associations (G/G, D/D, and D/G), according to previous criteria (PEDRAZZINI and SLAVUTSKY 1991). The number of association pairs (ASPs) was used as an integrated parameter for estimating the degree of SA (RAVIA et al. 1985). The frequency of cells with association complexes of three or more chromosomes was also recorded.

Kruskal-Wallis test was employed for nonparametric comparisons of the raw Ag-NORs and SA data. Correlations were studied by the Kendall rank correlation coefficient, $r_k$.

## Results

Table 1 shows age, sex, mean frequencies of Ag-NORs, and the percentage of Ag-NOR positive cells in BM cultures from controls and patients. Total mean number of Ag-NORs per metaphase, expressed as $\bar{X} \pm SE$, was found to be significantly increased in patients ($7.20 \pm 0.25$) compared with the controls ($5.40 \pm 0.16$) ($p < 0.002$). The distribution of Ag-NORs was further analyzed to look into variations with respect to D and G group chromosomes. Statistical analysis revealed that the increased frequency was due to the D group ($p < 0.002$). Moreover, a significantly higher percentage of Ag-NOR(+) cells in the patients ($71.7 \pm 3.9$) than in the controls ($48.0 \pm 7.8$) ($p < 0.02$) was seen. Correlations of Ag-NORs with aging in both groups were analyzed. A significant decrease with age in the percentage of Ag-NOR(+) cells was only found for controls ($r_k = -0.68$; $p < 0.035$) (Fig. 2).

The number of different SA types between acrocentric chromosomes, the mean of ASPs per cell and the frequency (%) of cells with different num-

*Table 1.* Frequency of Ag-NORs in controls and patients

| Sample | Age/Sex | Mean of Ag-NORs | | | No. cells scored | % Ag-NOR(+) cells |
|---|---|---|---|---|---|---|
| | | D Group | G Group | Total | | |
| *Controls* | | | | | | |
| 1 | 22/M | 3.20 | 2.53 | 5.73 | 200 | 75.0 |
| 2 | 24/M | 3.07 | 2.73 | 5.80 | 178 | 64.6 |
| 3 | 28/F | 3.73 | 1.57 | 5.30 | 640 | 49.6 |
| 4 | 29/M | 2.13 | 2.83 | 4.97 | 286 | 49.1 |
| 5 | 38/F | 2.80 | 1.97 | 4.77 | 254 | 22.0 |
| 6 | 40/F | 2.93 | 2.87 | 5.80 | 355 | 20.0 |
| 7 | 40/M | 3.50 | 1.93 | 5.43 | 307 | 56.0 |
| $\bar{X} \pm SE$ | | $3.05 \pm 0.20$ | $2.35 \pm 0.20$ | $5.40 \pm 0.16$ | | $48.0 \pm 7.8$ |
| *Patients* | | | | | | |
| 1 | 17/M | 4.60 | 2.37 | 6.97 | 250 | 79.2 |
| 2 | 32/M | 5.37 | 2.90 | 8.27 | 1100 | 68.8 |
| 3 | 44/F | 4.13 | 2.40 | 6.53 | 260 | 60.2 |
| 4 | 60/M | 4.70 | 3.23 | 7.93 | 616 | 86.0 |
| 5 | 67/F | 4.53 | 2.00 | 6.53 | 768 | 67.1 |
| 6 | 74/M | 4.00 | 3.07 | 7.07 | 363 | 60.0 |
| 7 | 75/M | 4.30 | 2.83 | 7.13 | 366 | 80.5 |
| $\bar{X} \pm SE$ | | $4.52 \pm 0.17$** | $2.69 \pm 0.17$ | $7.20 \pm 0.25$** | | $71.7 \pm 3.9$* |

* Significant differences with respect to controls, $p < 0.02$
** Significant differences with respect to controls, $p < 0.002$

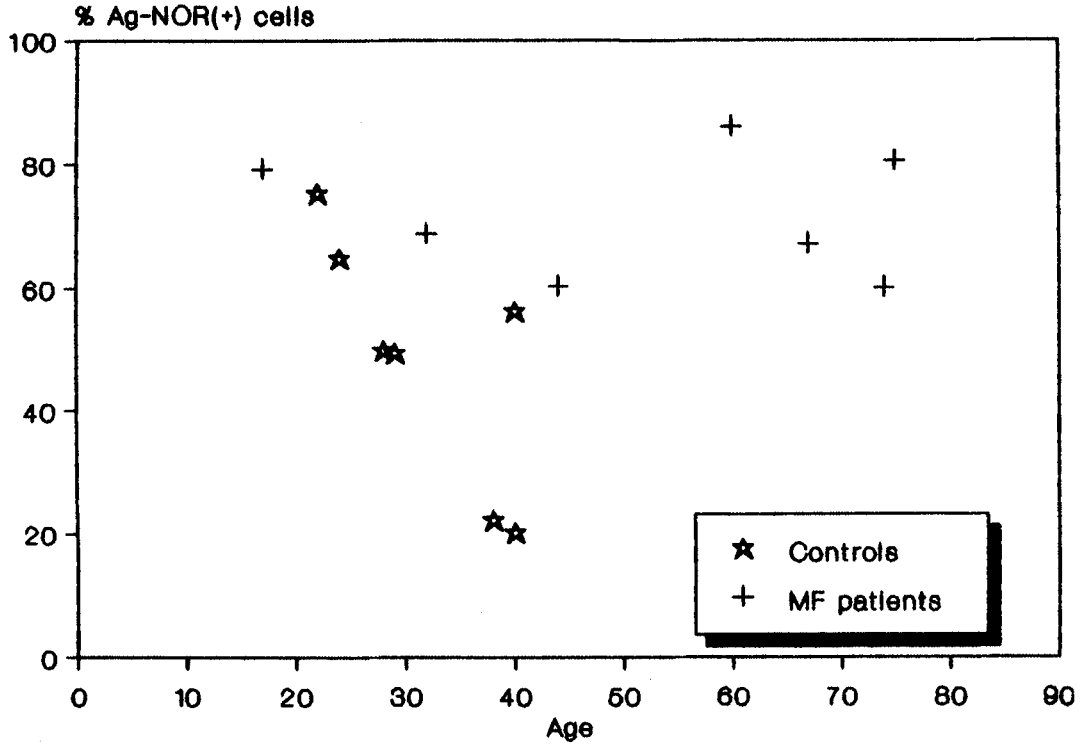


**Fig. 2.** Scatter diagram showing the correlation between the percentage of Ag-NOR(+) cells and aging. There is a significant negative correlation for controls ($r_k = -0.68$, $p < 0.035$).

*Table 2.* Satellite Association in controls and patients

| Sample | Type of SA | | | Mean of ASPs cell | Frequency (%) of cells with | | | |
|---|---|---|---|---|---|---|---|---|
| | G/G | D/D | D/G | | 0 ASPs | 1–2 ASPs | 3 or more ASPs | Complexes of 3 or more chromosomes |
| *Controls* | | | | | | | | |
| 1 | 0.03 | 0.07 | 0.10 | 0.20 | 83.3 | 16.7 | 0.0 | 0.0 |
| 2 | 0.00 | 0.07 | 0.13 | 0.20 | 80.0 | 20.0 | 0.0 | 0.0 |
| 3 | 0.00 | 0.13 | 0.10 | 0.23 | 80.0 | 20.0 | 0.0 | 0.0 |
| 4 | 0.13 | 0.03 | 0.13 | 0.30 | 76.7 | 23.3 | 0.0 | 0.0 |
| 5 | 0.00 | 0.03 | 0.17 | 0.20 | 83.3 | 16.7 | 0.0 | 0.0 |
| 6 | 0.07 | 0.03 | 0.27 | 0.37 | 66.7 | 33.3 | 0.0 | 0.0 |
| 7 | 0.00 | 0.07 | 0.23 | 0.30 | 80.0 | 16.7 | 3.3 | 0.0 |
| $\bar{X} \pm SE$ | $0.03 \pm 0.02$ | $0.06 \pm 0.01$ | $0.16 \pm 0.02$ | $0.26 \pm 0.02$ | $78.6 \pm 2.2$ | $20.9 \pm 2.3$ | $0.5 \pm 0.5$ | $0.0 \pm 0.0$ |
| *Patients* | | | | | | | | |
| 1 | 0.03 | 0.10 | 0.07 | 0.20 | 80.0 | 20.0 | 0.0 | 0.0 |
| 2 | 0.07 | 0.30 | 0.57 | 0.93 | 50.0 | 40.0 | 10.0 | 6.7 |
| 3 | 0.07 | 0.07 | 0.07 | 0.20 | 80.0 | 20.0 | 0.0 | 0.0 |
| 4 | 0.23 | 0.37 | 0.53 | 1.13 | 40.0 | 43.3 | 16.7 | 10.0 |
| 5 | 0.00 | 0.07 | 0.20 | 0.27 | 80.0 | 20.0 | 0.0 | 0.0 |
| 6 | 0.00 | 0.00 | 0.33 | 0.33 | 66.7 | 33.3 | 0.0 | 0.0 |
| 7 | 0.23 | 0.30 | 0.50 | 1.03 | 40.0 | 46.7 | 13.3 | 3.3 |
| $\bar{X} \pm SE$ | $0.09 \pm 0.04$ | $0.17 \pm 0.05$ | $0.32 \pm 0.08$ | $0.59 \pm 0.16$ | $62.4 \pm 7.1$ | $31.9 \pm 4.5$* | $5.7 \pm 2.8$ | $2.9 \pm 1.5$ |

* Significant differences with respect to controls, $p < 0.05$

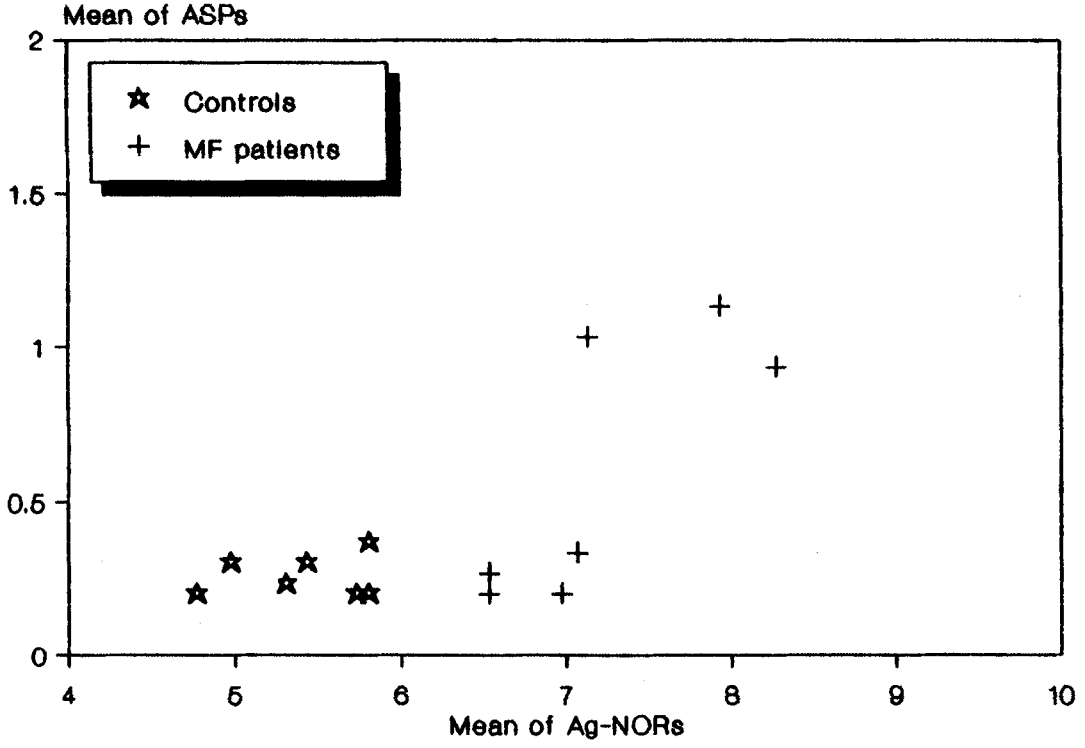


**Fig. 3.** Scatter diagram showing the correlation between the mean of ASPs and the mean of Ag-NORs per cell. There is a significant positive correlation for MF patients ($r_k = 0.65$, $p < 0.05$).

bers of ASPs and with complexes of 3 or more chromosomes from controls and patients, are shown in Table 2. MF patients showed an increase in the mean of ASPs per cell ($0.59 \pm 0.16$) compared to controls ($0.26 \pm 0.02$), but this was not statistically significant, probably due to the wide dispersion of the patient data. The analysis of the distribution of frequencies of cells with different numbers of ASPs, showed a significant increase in the percentage of cells with 1–2 ASPs in patients ($31.9 \pm 4.5$) with respect to controls ($20.9 \pm 2.3$) ($p < 0.05$), and a trend to a decrease in the percentage of cells without ASPs. Furthermore, the analysis of complexes of 3 or more chromosomes showed that the control group did not have cells with complexes, whereas in the patients they were observed in $2.9 \pm 1.5$ % of cells. A positive correlation between the number of Ag-NORs and the mean of ASPs per cell was found for patients ($r_k = 0.65$; $p < 0.05$) (Fig. 3). No significant relation was found for controls.

## Discussion

Silver staining of BM metaphases has been studied in several leukemias, but to the best of our knowledge there are no references about chronic lymphoproliferative disorders. Our findings clearly demonstrate that consistent differences exist between MF and normal bone marrow cells with respect to their silver-stained NORs and their involvement in SA. We have previously studied NOR activity in PBLs of MF patients (PEDRAZZINI and SLAVUTSKY 1991) and the results were different to those observed in BM cells. No difference in the total frequency of Ag-NORs and a significant increase in the degree of SA in PBLs, were found.

Normal BM metaphases of our control group exhibited a mean of 5.40 Ag-NORs per cell, quite similar to those of 5.10 and 4.81 reported by MAMAEV et al. (1985) and SATO et al. (1986), respectively. Analogously, a 48 % of Ag-NOR(+) cells was found, also in accordance to those previously reported (MAMAEV et al. 1985; SATO et al. 1986; ARDEN et al. 1985, 1989). Furthermore, a reduction in the staining of NORs in function with age was observed, probably related with a decrease in the proliferative activity (VAZIRI et al. 1994) or with a progressive inactivation of rRNA genes with aging (DENTON et al. 1981; BUTLER and LANE 1989).

On the other hand, a statistically high level in the mean number of Ag-NORs and a significant increase in the percentage of Ag-NOR(+) cells, without dependence with age, were found in our MF patients. It has been demonstrated that in BM metaphases silver staining is an indicator of the maturation/differentiation state. A gradual suppression of rDNA transcription during terminal maturation has suggested characteristic NOR staining profiles for different BM cell types (REEVES et al. 1984). This has been proved in studies carried out on BM interphase cells, where the number of Ag-grains in nucleoli decreases when the maturation takes place and the proliferative activity declines (MAMAEV et al. 1985; NIKICICZ and NORBACK 1990; GROTTO et al. 1991). Moreover, an overall increase in the methylation level during cell differentiation in different tissues was reported (MUNHOLLAND and NAZAR 1987). Thus, our results suggest the presence of cells with a certain degree of immaturity and a high proliferation activity in the BM of MF patients.

In relation to the satellite associations of acrocentric chromosomes, our control group showed between 0.20 and 0.37 ASPs per cell, with a 21.4 % of cells with SA, comparable with a 8 to 20 % reported by REEVES et al. (1982), and in no case cells were seen with complexes of 3 or more associated chromosomes. The frequency of SA is known to be positively correlated with the degree of NOR staining in stimulated lymphocytes (MILLER et al. 1977). So, the low degree of SA of normal BM cells could be associated with the low number of Ag-NORs in this tissue.

Another explanation would be related with the length of time a cell spends in interphase. Analysis on stimulated lymphocytes, cultured during 72 h in presence of BUdR to assess cell cycle kinetics simultaneously with SA, has demonstrated that cells in the first cycle have higher number of metaphases with SA than have those in second or third cycle (SIGMUND et al. 1979; SOZANSKY et al. 1985). Likewise, a relatively short cell cycle time, i.e., less than 20 h, has been observed by means of the analysis of normal BM cells in presence of BUdR (ABE et al. 1979). So, it is likely that short interphases account for the low degree of associations in normal BM cells.

On the contrary, MF patients displayed a wide distribution, with a nonsignificant increase in the mean of ASPs. Simultaneously were seen a significant increase in the percentage of cells with 1–2 ASPs and a trend to an increase in cells with 3 or

more ASPs. We could also observe that patients had a lower percentage of cells without satellite associations than controls. When patients were individually analyzed, three of them (cases 2, 4 and 7), who had the highest numbers of Ag-NORs, exhibited particularly high values of ASPs per cell, an increment in the percentage of cells with SA and the presence of association complexes of 3 or more chromosomes, demonstrating a further important NOR activity. These findings suggest that cells with more rDNA activity would have a major possibility to produce SA and suggest an increase in the cell cycle duration in the BM cells of MF patients.

It has been assumed that in the erythroid and granulocytic precursors of BM, the functional contacts between Ag-NOR chromosomes are not so close as those in lymphoid cells, as evidenced by the number of nucleoli (MAMAEV and MAMAEVA 1990). This is in good agreement with a higher association capacity of the acrocentric chromosomes in PHA-stimulated lymphocytes compared to that in BM cells of the same individuals (GRABOVSKAYA et al. 1986). Likewise, it can be seen when our results on PHA-stimulated peripheral blood cultures from MF patients and controls (PEDRAZZINI and SLAVUTSKY 1991), are compared with the data here reported.

Furthermore, BM examination of our MF patients displayed lymphocytosis, and the analysis of PBLs in a few of them, using MAC (Morphology-Antibody-Chromosome) methodology (TEERENHOVI et al. 1984), showed that the abnormal proliferating cells were T lymphocytes (unpublished data).

Our present results suggest the particular involvement of BM in MF patients. It may be associated with a certain degree of immaturity, a high proliferative activity and modifications of the growth rate of this tissue.

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