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GLUTATHIONE-S-TRANSFERASE ACTIVITY AND MULTIDRUG RESISTANCE PHENOTYPE IN CHRONIC LYMPHOCYTIC LEUKEMIA: DO THEY HAVE ANY CLINICAL RELEVANCE?

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ABSTRACT

Background. **Lymphocytes from patients affected by B-cell chronic lymphocytic leukemia (B-CLL) have frequently been shown to be positive for the multidrug resistance (MDR) phenotype. However, this phenotype does not seem to be responsible for the resistance to alkylating agents usually employed in the management of CLL.**

Methods. **Lymphocytes from 42 patients were evaluated by flow cytometry for P-170 expression and by spectrophotometry for glutathione-S-transferase (GST) activity.**

Results. **Our findings show that GST is not related to any clinical parameter but is increased in treated patients. Conversely, 85% of patients were positive for P-170 and this was related to the percentage of CD5/CD19-positive lymphocytes. CD5/CD19-negative patients were also negative for P-170. MDR was not related to any clinical parameter evaluated nor to GST activity in lymphocytes.**

Conclusions. **MDR is constitutively expressed in B-cell chronic lymphocytic leukemia and seems to be related to a CD5/CD19 B-CLL phenotype. The increase of GST activity in treated patients is statistically significant ($p < 0.005$).**

Key words: multidrug resistance, P-170 glycoprotein, glutathione-S-transferase, chronic lymphocytic leukemia, chlorambucil

Chronic lymphocytic leukemia (CLL) is a neoplastic disease that is usually sensitive to chlorambucil (CLB), a bifunctional alkylating agent. While most patients show a good response to this drug, the development of resistance may hamper further treatment. Lymphocytes from CLL have been found[1] to express the P-170 glycoprotein on their membrane,[2] which may be responsible for the multidrug resistance (MDR). In spite of some discrepancies in the reported percentages of positive patients, possibly due to methodological differences,[3] there is widespread agreement that nearly 100% of such patients can be shown to be MDR positive by using both monoclonal antibodies and molecular biology techniques.[4,5] Apart from the biological relevance and possible implications in B cell malignancies, such a broad positivity could not play a role in resistance to the conventional therapy of B-CLL, which is based on the administration of alkylating agents.

Regarding resistance to these latter agents, several mechanisms have been proposed, including increased DNA repair,[6] increased glutathione (GSH) content and glutathione-S-transferase (GST) activity.[7] It has been reported that there is no difference in GST activity between normal B and T lymphocytes.

Characterization of GST in lymphocytes from CLL has revealed that there are no significant differences in isoenzyme composition between CLL and normal lymphocytes. However, lymphocytes from resistant patients showed a two-fold increase in GST activity compared to

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untreated patients lymphocytes.[8] The role of GST in chlorambucil resistance was evaluated in the resistant cell line N50-4, where both GST inhibitors ethacrynic acid (EA) and indomethacin were partially able to reverse CLB resistance.[9]

The presence of mdr1 gene expression has been found to correlate significantly with expression of the glutathione-S-transferase (GST) gene and with response to chemotherapy in multiple myeloma.[10]

Moreover, transformation of rat liver epithelial cells with v-H-ras causes expression of MDR1, GST-π and increased resistance to cytotoxic chemicals.[11] On the basis of these findings we decided to study P-170 glycoprotein expression and GST activity in lymphocytes from B-CLL patients in order to evaluate the possibility of a relationship between these two mechanisms of resistance.

Materials and Methods

Forty-two consecutive patients, median age 69 (range 39- 80), 28 male and 14 female, were included in this study.

The diagnosis of B-CLL was formulated on the basis of conventional parameters that included blood counts, peripheral blood and bone marrow examination, immunological typing by cytofluorimetric analysis, ultrasound and X-ray scanning to evaluate organ and node enlargement. Staging was performed according to the Rai[12] and Binet[13] systems.

Results of the Rai staging system showed that 4 patients were at stage 0, 17 at stage I, 8 at stage II, 3 at stage III and 10 at stage IV. The Binet staging system placed 21 patients at stage A, 8 at stage B and 13 at stage C. In this report results were analyzed according to Rai's staging.

Twenty-two patients had been treated before the study and 20 were previously untreated. Among the pretreated patients, 11 were receiving therapy at the time of the study.

CLB had been administered to all treated patients. Of the 22, 19 were evaluable for response; only four were considered non responders, since their lymphocyte number either failed to decrease or even increased after 1 month of chemotherapy.

Enzyme measurements

Mononuclear cells were purified by density separation, counted and pellets were stored at –80°C until examination.

Samples were then lysed by sonication in 1 mL each of potassium phosphate buffer 0.01 M pH 7.6, KCl 0.15 M, EDTA 1 mM. The supernatant obtained after centrifugation (100,000 × g for 45 min at 4°C) was used for the enzyme measurements.

Glutathione-S-transferase activity was measured with CDNB as substrate according to Habig[14] and expressed as nmoles of adduct formed/min per 10^7 cells or per mg of proteins.

Flow cytometry

Mononuclear cell suspensions were fixed by periodate lysine paraformaldehyde (PLP)[4] for 15 min at 4°C and then incubated for 15 min at room temperature with the monoclonal antibody JSB-1, directed against an internal epitope of the P-glycoprotein[15] (the antibody was diluted 1:10 in saponine 0.02% in PBS). After three washings, cell suspensions were incubated for an additional 15 min at room temperature with a fluoresceine-labelled $F(ab)_2$ fragment of rabbit anti-mouse immunoglobulin. After further washing, flow cytometry was carried out using a fluorescence-activated cell sorter (Facstar Becton and Dickinson). Appropriate controls were performed by substituting an indifferent mouse immunoglobulin for the primary antibody.

Since 8.74±5% positive lymphocytes (mean fluorescence intensity 28.8) were found in the mononuclear cell population from 10 healthy donors, we fixed 18% JSB-1+lymphocytes as the cut-off for P-170 positive patients.

The significance of differences among groups was evaluated by Student's t-test.

Results

GST activity in the entire group of patients was 29.89±15.14 (range 7.8-80.6) nmoles/min/10^7 cells (Table 1) or 165.16±86.49 (range 48.8-433) nmoles/min/mg of protein, and this was not significantly different from the GST activity found in the mononuclear cells from healthy donors

Table 1. Description of patients. Staging according to the Rai classification is reported. Nineteen patients had never been treated. Those evaluable for sensitivity to treatment are reported as responsive (R) or unresponsive (NR). GST activity is expressed as nmoles of adduct formed/min per 10^7 cells. P-170 is evaluated as the percentage of JSB-1- reactive cells.

patient	*age*	*sex*	*stage*	*response*	*GST*	*p-170*
AA	66	m	I		18.3	
AS	60	m	II	R	47.4	34
BP	49	f	I		9.2	66
BR	71	f	IV	NR		56
BG	75	m	IV	R	41.2	27
BR	65	f	IV	NR	20.2	92
BE	73	m	I	R	28.1	40
BAM	65	f	II	R	42.1	72
BG	41	m	II	R	20.7	11
CM	51	f	IV	R	31.7	25
CR	54	m	0		41.8	70
CG	58	m	I	R	14.7	41
CP	67	m	II		9.9	73
DB	60	m	II		15.2	54
DCI	74	m	III	NR	70.1	90
DL	67	f	II		43.5	
FA	79	f	IV	R	19.6	69
FE	69	f	I		21.4	45
FR	41	m	IV	R	53.8	42
FG	67	m	I	R	80.6	50
GA	39	m	IV	R	11.3	69
GG	50	m	0		35.4	
LG	61	m	III	R	26.0	60
LF	60	m	I		18.8	10
MI	72	f	I	R	21.0	13
MV	50	m	IV	R	22.8	80
MC	62	f	III	R	24.3	
MP	68	m	I	R	58.2	
NC	79	f	I	R	39.1	36
OO	80	m	I		27.5	
OR	70	f	0		26.9	50
PC	65	m	II	R	40.9	59
PA	51	m	II	R	30.0	61
PB	68	m	I		21.0	83
PR	70	m	I		30.9	51
PL	64	m	I		30.0	17
PF	51	m	IV		7.8	9
RR	72	f	0		14.3	
SG	60	m	I		22.4	79
SB	71	m	IV	NR	29.7	87
SR	55	m	I		29.6	67
SR	53	f	I		45.9	54

(28.7 nmols/min/10^7 cells; range 10.3-41.2). No difference was found between the GST activity of leukocytes from patients in stages 0-I and that of white cells from patients in stages II-IV, whether previously treated or untreated. Leukocytosis was not related to GST activity.

Treated patients had significantly ($p<0.005$) higher activity (39.18±17.9 nmols/min/10^7cells) than untreated ones (24.71±11.57 nmols/min/10^7 cells), but we observed no differences between patients evaluated during treatment and those who had been pre-treated, nor were there any between patients who had been treated in the last six months and those who had stopped treatment at least one year before. Only four patients failed to respond, so no statistical evaluation concerning GST activity in responder and non-responder patients was carried out.

Thirty of 35 patients (85 %) (Table 1) were P-170 glycoprotein positive (Figure 1). We could not find any relationship between the percentage of P-170-positive lymphocytes and stage, leukocytosis, treatment or any other clinical parameter evaluated.

Two patients did not express the CD5/CD19 phenotype and proved to be P-170 negative. Moreover, a linear correlation was found between the percentage of lymphocytes expressing CD5/CD19 and that of JSB-1-positive lymphocytes ($r= 0.453$; $p= 0.023$). No relationship was observed between the percentage of P-170-positive lymphocytes and GST activity.

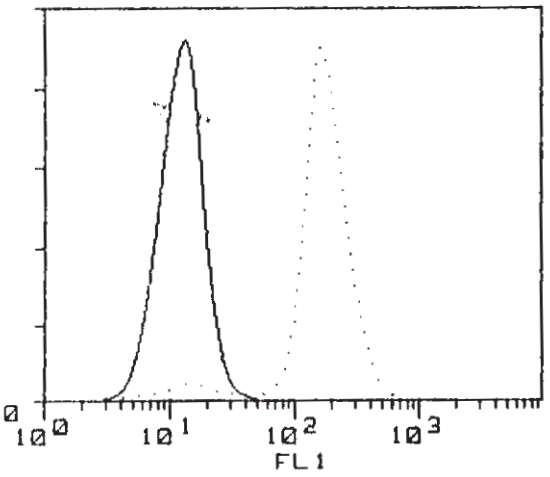

Figure 1. The overwhelming majority of cells are reactive to the monoclonal antibody JSB-1 (·····) compared to the control where JSB-1 has been substituted by an indifferent antibody (——).
Only a minor subpopulation of cells incubated with JSB-1 appears to be completely negative. Both the control test and the sample from the patient were incubated with the same secondary fluoresceine-conjugated antibody.

Discussion

Drug resistance is a relevant event in the management of CLL patients and new drugs such as fludarabine have recently been employed to overcome this problem.[16,17] Besides the many resistance mechanisms reported,[18] lymphocytes from CLL patients have frequently been found to express the P-170 glycoprotein on their membrane and this may be responsible for multidrug resistance.[1,3-5] For these reasons, we decided to evaluate GST activity (possibly related to resistance to alkylating agents[7,8]) and P-170 expression on a relatively large number of patients followed at our institution. GST was not significantly elevated in our patients and this activity was not related to any of the clinical or hematological parameters evaluated. The only increase we found was in patients treated with CLB versus untreated ones. It appears interesting to note that there was no difference between patients under treatment and those who had stopped therapy one year before. This observation suggests that either CLB is able to induce a permanent enzymatic increase, or that it selects a lymphocyte subpopulation carrying higher GST activity. Among our patients, only four failed to respond and it is impossible to conclude whether GST activity may be related to CLB resistance; however, it should be noted that some resistant patients showed normal GST activity, whereas some sensitive ones demonstrated very elevated GST activity. A possible role for GST in CLB resistance was previously reported,[8] and we were able to document the role of ethacrynic acid (a GST inhibitor) in reversing CLB resistance in a case with extremely elevated GST activity.[19] Thus it is tempting to suggest that GST activity itself will not predict resistance to CLB but that it may be responsible for resistance in some cases, possibly depending upon certain agents such as, for example, glutathione content.

In accordance with the literature, the overwhelming majority of CLL patients proved to be MDR positive. We did not find any relationship with stage or other clinical parameter. Since there were no differences between treated and untreated patients, we might exclude any role for therapy in inducing P-170 expression in this disease. On the basis of these findings, it is tempting to suggest that the MDR phenotype is a constitutive feature of B-CLL. This hypothesis agrees with a recent paper from Sparrow[20] that showed P-170 expression in a minor subpopulation (3%) of CD5/CD19-positive lymphocytes isolated from normal controls, suggesting that these cells could be the potential precursors of B-CLL cells. We found a significant correlation between the percentage of CD5/CD19-positive and P-170-positive lymphocytes in our patients. Moreover, lymphocytes from the two patients not expressing the CD5/CD19 phenotype were P-170 negative.

Thus only three of the reported patients who showed the common CD5/CD19 phenotype were considered negative by our test; however, it should be kept in mind that they expressed a low but consistent percentage of P-170-positive lymphocytes (10, 13, 17%, respectively) among cells expressing more than 80% of B-CLL immunological markers. Based on this, it appears appropriate to state that MDR is a constitutive marker of CD5/CD19 B-CLL. Neither GST nor MDR was related to any clinical parameter evaluated and neither appears to be related to drug resistance in these patients. Thus they are not useful for clinical purposes. We suggest that they should be evaluated in a selected number of resistant patients for research purposes.

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