Cyclophosphamide Boluses Induce Micronuclei Expression in Buccal Mucosa Cells of Patients with Systemic Lupus Erythematosus Independent of Cytochrome P450 2D6 Status

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ABSTRACT. ***Objective***. To assess the effect of cyclophosphamide (CYC) and the influence of CYP2D6 polymorphism on micronuclei expression in patients with systemic lupus erythematosus (SLE).

Methods. Micronuclei (MN) assays were performed in oral mucosa sweeps of 49 patients with SLE, receiving and not receiving CYC therapy, and 43 healthy controls. In patients receiving CYC the MN assay was repeated 14 days after CYC administration. SLE patients underwent determination of the CYP2D6 allele expressed in peripheral blood by polymerase chain reaction amplification. These results were compared with CYP2D6 distribution in a healthy population from the same geographic area. The results were analyzed by Mann-Whitney U and chi-square tests.

Results. Twenty-four patients with SLE received CYC boluses. The mean age of SLE patients was 32 ± 10 years; mean disease duration was 7 ± 6 years. Basal expression of MN was similar in the groups investigated and increased significantly in those exposed to CYC (CYC group 44%, no CYC group 5%, healthy controls 9%; $p = 0.001$). We found no association between the CYP2D6 allele expressed and MN induction. Poor metabolizers were overrepresented in SLE subjects as compared with 139 healthy controls ($p < 0.05$).

Conclusion. Genotoxicity, as assessed by the MN assay, is increased in patients with SLE after CYC boluses. The CYP2D6 allele expressed in SLE patients does not have a role in CYC induced chromosomal injury. (J Rheumatol 2004;31:1335–9)

Key Indexing Terms:
SYSTEMIC LUPUS ERYTHEMATOSUS GENOTOXICITY PHARMACOGENOMICS
CYP2D6 CYCLOSPHOSPHAMIDE IMMUNOSUPPRESSIVE THERAPY

Systemic lupus erythematosus (SLE) is a chronic, autoimmune disorder that may affect diverse organs and systems. Morbidity and mortality have improved for lupus patients during the last 15 years, possibly due to the extended use of immunosuppressive therapy; yet an increased risk of malignancy has been shown in several studies[1-4].

A number of patients with SLE develop major organ involvement requiring prompt and aggressive treatment. Rheumatologists all over the world use diverse regimens of high dose steroids and immunosuppressive therapy for several months[5]. Cyclophosphamide (CYC) is widely used to treat SLE nephropathy and other complications of the disease. Although useful, CYC treatment can cause serious short and longterm complications. Some of these may be dose related, and some others seem to be idiosyncratic and independent of dose[6]. Several reports have shown CYC increases the risk of cancer. This is well described in rheumatoid arthritis and Wegener's granulomatosis[7,8]. In rheumatoid arthritis malignancies occur in a dose-dependent relationship. As an example, cumulative doses exceeding 30 g present a 5% overall incidence of hematologic malignancies[9].

Among the difficulties in ascertaining the influence of CYC on cancer induction in patients with SLE are (1) a long latency period between exposure to the drug and appearance of malignancy, and (2) the formal causal association between CYC exposure and cancer induction. Patients with SLE frequently switch from one immunosuppressive drug to another or use a combination of them during the course of

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the disease, thus making it difficult to ascribe the appearance of the neoplasm to a specific drug.

The micronuclei (MN) test is an indirect and sensitive measure of chromosomal breakage or misaggregation used as a biologic marker of genotoxic exposure[10]. MN are chromatin-containing bodies that represent chromosomal fragments not incorporated during mitosis[11,12]. They result from chromosome breakage or interference with the mitotic apparatus, events thought to be related to carcinogenesis. This test is used in the evaluation of the genotoxic potential of diverse pharmacologic products and environmental hazards[13,14]. An increase in the number of MN is an indicator of the potential toxicity of the product evaluated.

Since CYC is processed in the body by the enzyme complex cytochrome P-450 (CYP450), it would be useful to determine the precise expression of diverse subtypes of CYP450 in each subject exposed to the drug to predict potential responses (toxic and nontoxic). Most of the CYC administered is processed by CYP2B6, although other enzymes of the complex are important. This is the case with CYP2D6, as evidenced in a report of severe toxicity due to CYC in a patient with no expression of the enzyme[15]. CYP2D6 metabolizes about 25% of all the drugs regularly used in humans, mainly antipsychotic and cardiovascular drugs[16]. The debrisoquine 4-hydroxylase (P4502D6) deficiency is the most widely studied human genetic defect of drug oxidation[17]. Those affected are deficient metabolizers of debrisoquine, sparteine, metoprolol, and several other drugs. In Caucasian populations, this phenotype occurs with a frequency of 5 to 10%. This metabolic defect usually reflects the absence of CYP2D6 protein caused by mutations in the CYP2D6 gene in affected individuals, which results in the poor metabolizer phenotype[18,19].

We assessed whether patients with SLE treated with CYC bolus express more MN than those not using it, and whether the polymorphism of CYP2D6 is associated with cellular toxicity, as assessed by MN test in patients receiving CYC bolus.

MATERIALS AND METHODS

This study was approved by the Ethics Committee of the Hospital de Especialidades, Centro Medico Nacional de Occidente, IMSS. Informed consent was obtained from all participants. We included consecutive patients fulfilling the American College of Rheumatology 1982 SLE classification criteria[20] and attending a tertiary care rheumatology department in Guadalajara, México. Those requiring CYC treatment because of SLE nephropathy class III or IV (World Health Organization classification) formed the intervention group. We included a second group of patients with SLE not taking immunosuppressives since we did not know if the disease per se could induce MN. We excluded patients with previous neoplasia and with other rheumatic diseases associated with SLE. We also excluded patients with environmental exposure to toxic substances. None of our patients had smoked tobacco since at least one month prior to the study. No patient was taking methylprednisolone boluses in combination with CYC administration.

Demographic information was obtained during a personal inteview and chart review using a structured questionnaire. Disease activity was assessed using the Mexican SLE Disease Activity Index (MEX-SLEDAI)[21]. All concomitant medications taken by the patient were registered. CYC boluses were administered at the hospital (500 mg to 1 g/m^2 of total body surface) and patients remained for medical monitoring for a few hours. The sample for the MN test was taken in the hospital before administration of the bolus; for those not receiving CYC, the test sample was obtained during medical consultation. Patients receiving CYC boluses had a second MN assay 14 days after the bolus.

MN sample preparation. Subjects were asked to rinse their mouth with water, and a polished slide was used to collect cells from the buccal mucosa of the right and left cheeks. The samples were spread directly into 2 separate precleaned slides. The smears were air dried and fixed in 80% methanol for 48 h, and stained using acridine orange[22]. After this procedure, the nucleus and micronuclei were stained in yellow and the cytoplasm in dark green. Slides were scored in an Olympus CX40 microscope equipped with epifluorescence. The scoring was done according to the criterion established by Tolbert, *et al*. Results are presented as MN/2000 cells[23,24].

Both evaluators of the slides were blinded to demographic and clinical information of the patients. The interobserver variability for these evaluations was 0.82. A group of 43 healthy volunteers within the age limits of our patients' sample was asked for mucosal samples for comparison as a control group.

CYP2D6 amplification. Genomic DNA was extracted from peripheral blood samples according to standard protocols. We recruited 139 healthy donors and 49 patients with SLE, all Mexicans living in Guadalajara and its surroundings.

The mutant G1934A change in intron 3 disrupts the pre-messenger ribonucleic acid (mRNA) splicing and results in deficient debrisoquine metabolism[25]. The presence of mutant G1934A of the CYP2D6 gene was determined by DNA amplification in a total polymerase chain reaction (PCR) volume of 25 µl containing 200 µM dNTP, 5 pm of primers, and 2.5 U Taq polymerase. We used the following primers: (exon 3 sequence) 5'-GCCTTCGCCAACCACTCCG-3', and (intron 4) 5'-AAATCCT-GCTCTTCCGAGGC-3'. These primers amplify a 334 bp fragment with G to A transition at the junction of 3/exon4, which in affected individuals is resistant to digestion with the restriction enzyme BstNI[26]. The PCR modification consisted of an initial melting temperature of 94°C (4 min) followed by 30 cycles of melting (94°C, 45 s), annealing (58°C, 1 min), and extension (72°C, 1 min). The samples were separated using 6% polyacrylamide gel electrophoresis (29:1) and were silver stained. Presence of the 334 bp band indicates homozygous poor metabolizers and the 230 + 104 bp indicated normal metabolizers or normal allele.

To assess differences between 2 continuous variables we employed Mann-Whitney U test. Chi-square test was employed for proportions.

RESULTS

All eligible patients agreed to participate. We included 49 patients, all receiving steroids; 24 were taking intravenous CYC. Demographic and clinical characteristics of patients with SLE are shown in Table 1. All patients were female; as expected, CYC users had more active disease than nonusers.

Table 2 shows the differences in the number of MN among CYC users, nonusers, and controls, as well as differences before and 14 days after CYC bolus. At baseline there were no significant differences in MN number among the 3 groups. However, there was a significant 3-fold increase 14 days after CYC administration. More patients in the CYC bolus group had ≥ 1 SD increase in MN expression compared with controls and with patients not taking bolus CYC (44% vs 9% vs 5%, respectively; p = 0.001).

Table 1. Differences between patients with SLE.

	Receiving CYC	Not Receiving CYC	p
Age, yrs	32 ± 10	30 ± 10	0.47
SLE duration, yrs	7 ± 6	7 ± 6	0.89
MEX-SLEDAI score	6.26 ± 3.11	1.91 ± 4.1	< 0.001
Prednisone, mg/24 h	11 ± 8	12 ± 6	0.87

MEX-SLEDAI: Mexican SLE Disease Activity Index.

Table 3 shows frequency distribution of CYP2D6 status in patients with SLE and in a sample of 139 healthy Mexican subjects. We found an increase in the proportion of poor metabolizers in the SLE group in comparison with healthy subjects from the same population, and a lower proportion of heterozygous subjects. We found no association between the diverse alleles of CYP2D6 expressed and MN test findings in our patients.

DISCUSSION

CYC is metabolized along 2 alternative pathways through the cytochrome P450 enzymatic system in the liver[27]. It can be 4-hydroxylated and ultimately decomposed to yield phosphoramide mustard, which is the cytotoxic metabolite, or it can be N-dechloroethylated. In this last case, 2 products result: dechloroethyl-cyclophosphamide and chloroacetaldehyde, which are formed in equimolar amounts. Dechloroethyl-cyclophosphamide is devoid of cytotoxic activity, but chloroacetaldehyde is associated with various serious toxicities, such as urinary tract toxicity and neurotoxicity. These 2 alternative metabolic pathways of CYC are catalyzed by distinct subsets of liver P-450 enzymes. So far, CYP 2A6, 2B6, 3A4, 3A5, 2C9, 2C18, and 2C19 have been found to exhibit 4-hydroxylase activity in CYC metabolism in humans, although 2B6 displays the highest activity[28]. In rat models, CYP 2B and 2C are also the major catalysts of 4-hydroxylation, and CYP3A is the major enzyme responsible for N-dechloroethylation identified to date.

Since the report by Gustafsson, *et al*, it is considered that CYP2D6 can be important in high-dose CYC challenges in patients with null or defective enzyme activity[15,29]. Because patients with SLE nephritis are exposed to high doses of CYC over several months in accord with the recommended treatment for this condition, it was important to investigate the association between the CYP2D6 alleles expressed and the potential toxic response to the drug.

To our knowledge, there is only one previous report assessing MN expression in patients with SLE receiving CYC. In a small sample, the authors report an increase of MN expression in peripheral blood lymphocytes in comparison with those SLE patients who had not yet received any medical treatment[30]. From these and our own results it is clear that SLE by itself does not increase MN expression. This finding is in clear contrast to other rheumatic diseases, such as systemic sclerosis and rheumatoid arthritis[31,32]. The precise mechanism by which these autoimmune diseases present with a high basal MN expression, and by which SLE lacks this finding, is still unclear. Also, the clinical significance of this finding must be given its proper weight over time.

We know of no previous evaluation of the expression of MN in buccal mucosal cells in patients with SLE receiving CYC, although the MN assay is an easy and inexpensive method for monitoring drug toxicity in other rheumatic diseases.

With our results, we confirm that MN induction by CYC is present not only in peripheral blood but also in other tissues, indicating the severe toxicity of the drug. We are unaware of previous evaluations of MN expression in response to cytotoxic treatment in patients with SLE in association with specific CYP450-enzyme complex expression. It is logically supposed that the prolonged exposure to the drug provoked by the slow conversion to nontoxic products by some CYC enzyme complex members can induce greater MN expression in diverse tissues.

According to our results, MN expression seems to

Table 2. Changes in micronuclei expression induced by CYC boluses.

Micronuclei Expression	SLE with CYC	SLE without CYC	Healthy Controls	p
At baseline	1.26 (± 1.23)	0.86 (± 1.08)	1.34 (± 1.37)	NS
14 days after treatment	3.41 (± 2.17)	—	—	< 0.001

Table 3. CYP2D6 expression in Mexican SLE patients and healthy subjects.

Genotype CYP2D6 (G1934A)	SLE Patients, n (%)	Healthy Subjects, n (%)	p, Chi-square
A1934, normal metabolizer	33 (67.6)	48 (35)	
G1934, poor metabolizer	9 (18.9)	10 (7)	< 0.05
A1934/G1934, heterozygous	7 (13.5)	81 (58)	
Total	49 (100)	139 (100)	

depend only on the exposure to CYC. The CYP2D6 allele expressed in patients with SLE does not seem to have a role in CYC induced chromosomal injury.

As expected from published reports on CYP2D6, the poor metabolizer phenotype represents the lower proportion in our population[33]. It is especially important to compare the expected frequencies with healthy controls coming from the same population to ensure a certain degree of homogeneity in the genetic background analyzed, since diverse genetic backgrounds are expected to express different proportions of enzyme activity[34].

We found a significantly higher proportion of poor metabolizer phenotype in patients with SLE as compared with 139 healthy controls. The poor metabolizer phenotype has been associated with a wide range of diseases, including Parkinson's disease[35] and cancer[36]. It has been associated with SLE as well; however, its precise participation in the pathogenesis of the condition has not been established[37]. Although our sample and the type of study design do not allow us to make any further inferences in this respect, the possible participation of this phenotype in the pathogenic process leading to autoimmunity should be explored in further investigations. CYP2D6 has been proposed as associated with disease susceptibility in other possibly autoimmune conditions such as scleroderma[38] and ankylosing spondylitis[39], but not in other rheumatic diseases[40].

We did not find an association between CYP2D6 polymorphism and clinical variables in SLE, such as disease duration or systems affected. No variations in the results are expected due to the age of patients or to disease duration, since these factors are not known to modify the expression of the diverse components of cytochrome P450. This is in agreement with results presented by other groups[41,42]. Since the selection of the patients was based on the presence of severe lupus nephropathy (e.g., requiring CYC boluses), an interesting explanation for the augmented frequency of the poor metabolizer phenotype in our patients is the association with this clinical manifestation. Again, and due to the specific design of this study, no further conclusions can be drawn from our results.

Other medications frequently used in SLE during acute nephropathy could increase the toxicity expressed by CYC through the MN assay. Special consideration should be given to other cytotoxic drugs and cyclosporine. Currently, no information exists on this topic. This is an important issue, since the recommended treatments in most rheumatic diseases include various regimens of cytotoxic drugs for long periods of time. In longterm CYC treatments, some of the potential adverse events such as infertility can be prevented by manipulation of the hormonal state of the patient. To date, there is not an accepted method to prospectively evaluate the risk associated with these treatments with respect to developing malignancies after exposure. The MN assay is an easy and inexpensive method to monitor the potential toxicities of diverse treatments employed in SLE and other autoimmune conditions. It can be performed in diverse biologic samples, such as peripheral blood or epithelia. The interesting potential of the MN test in prospective evaluation is to define subgroups of patients, possibly by their exacerbated production of MN, with a higher risk of developing malignancies associated with these regimens. The longterm potential of the MN assay to predict and prevent serious adverse drug events associated with the known toxicities of immunosuppressive treatments employed in rheumatic autoimmune conditions should be evaluated.

Micronuclei expression is increased in patients with SLE after exposure to cyclophosphamide boluses with no influence exerted by the CYP2D6 allele expressed. Since it is an inexpensive and reproducible method to assess cellular damage, it should be measured and followed prospectively to assess its utility as a predictor of further drug complications in SLE.

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