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Genotoxicity assessment using micronuclei assay in rheumatoid arthritis patients

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ABSTRACT

Objectives: *This study investigated whether: (i) rheumatoid arthritis (RA) patients have more micronuclei (MN) than healthy controls; (ii) methotrexate (MTX) treated RA patients have more MN than those not using MTX, and (iii) folic acid supplementation decreases the number of MN in MTX treated patients.*

Methods: *MN assays were performed in oral mucosa sweeps of 50 consecutive MTX treated RA patients, 30 consecutive RA patients not receiving MTX and 39 healthy controls. MTX treated RA patients were then randomly placed in a cross-over design to receive folic acid supplementation, and MN assays were repeated after 6 weeks.*

Results: *The MTX-RA patients had a mean age of 46 ± 10 yrs and a mean disease duration of 12 ± 9 yrs; 80% were women. The MTX dose range was 8.7 ±1.5 mg/week and the mean duration of use was 16 ± 18 months. In the non-MTX RA group, the mean age was 48 ± 14 yrs, the mean disease duration was 13 ± 9 yrs, and 87% were women. At baseline, the number of MN were significantly higher in RA patients as compared with controls (3.31 ± 2.3 vs 0.8 ± 0.8, $p < 0.001$). No difference in MN numbers was observed between users and non-users of MTX. Folic acid supplementation did not decrease the MN number in the MTX treated RA patients.*

Conclusions: *Genotoxicity, as assessed by the MN assay, is increased in RA patients. These results suggest that genotoxicity is associated with RA itself and not with MTX use. Folic acid supplementation had no effect on the number of MN.*

Introduction

Several studies have estimated the risk of malignancy in rheumatoid arthritis (RA) patients. It seems that the distribution of site-specific cancers in RA is skewed compared to the general population; the incidence of lymphomas and bladder cancer is increased, while the incidence of cancer of the digestive organs is decreased (1,2). Germane to any examination of RA and malignancy is the cancer-sparing or cancer-promoting potential of RA therapies and the influence of RA by itself. Experimental studies and human cohorts have shown a reduced risk of colorectal, lung and breast cancer in association with the use of nonsteroidal anti-inflammatory drugs (NSAIDs). In contrast, a number of studies have found an increased risk of malignancy in RA patients treated with disease-modifying antirheumatic drugs (DMARDs). For instance, azathioprine and cyclophosphamide have been reported to increase the risk of lymphoprolipherative malignancies in RA patients (3-5). Methotrexate may also be associated with hemopoietic malignancies, although the literature consists predominantly of case reports. In a case-control study, Williams *et al*. (6) found that RA patients with leukemia or lymphoma showed a trend toward increased prior methotrexate or azathioprine use compared with matched RA controls without leukemia or lymphoma.

There are two main problems in ascertaining the influence of specific DMARDs on cancer induction. The first is the long latency period between exposure to a given DMARD and the development of malignancy. The other is that RA patients frequently switch from one DMARD to another, or use a combination of them during the course of their RA, thus making it difficult to sustain a causal inference.

The micronuclei (MN) test is an indirect and sensitive measure of chromosomal breakage or missegregation and has received increased attention as a sensitive biologic marker of genotoxic exposure (7). This test has been applied to the biological monitoring of human populations exposed to mutagenic and carcinogenic agents. MN are extranuclear bodies composed of chromosomal fragments or entire chromosomes that were not incorporated into daughter nuclei at mitosis (8). They result from chromosome breakage or interference with the mitotic apparatus, events thought to be related to carcinogenesis. Exfoliated cells from the intermediate and superficial layers of the epithelium have been widely used in cytology to detect abnormal morphology, premalignant changes, and cancer (9). The technique used in this study involves

examination of epithelial smears to determine the prevalence of cells containing micronuclei, and it has been used to evaluate the effect of exposure to different xenobiotics which are capable of inducing cancer (10).

Therefore, the MN test in exfoliated cells from oral mucosa can be used as a tool in studies aimed at assessing the potential genotoxicity of different drugs used to treat rheumatic diseases. Studies *in vitro* and in experimental animals have reported that MTX may induce MN in a dose dependent manner (11, 12), and that folinic acid supplementation reduces the cytogenetic damage induced by MTX (13). Yet to the best of our knowledge, MN induction has not been studied in RA patients using MTX.

The aims of this study were to assess: (a) whether RA patients have more MN than healthy controls, (b) whether MTX treated RA patients have more MN than those not using MTX, and (c) whether folic acid supplementation decreases the number of MN in these patients.

Materials and methods

We included consecutive RA patients fulfilling the ACR criteria (14) and attending a secondary care outpatient rheumatology clinic in Guadalajara, Mexico. At baseline all patients had a prospective rheumatologic assessment. Demographic data, disease characteristics and comorbid conditions were documented using a structured questionnaire and chart review. Patients with a history of any oncologic disease or with other diseases (such as diabetes mellitus, hypertension, thyroid diseases, *etc.*) were not included.

Two groups were assembled. The first group was composed of 50 consecutive RA patients receiving MTX but not folic acid supplementation. The second group consisted of 30 RA patients who had never used MTX or azathioprine. All patients underwent an MN assay at their first visit. Group One (RA-MTX) was then randomly placed in one of two subgroups. In subgroup A (RA-MTX/Folic Acid) patients were given folic acid supplementation (5 mg p.o. per day for 6 weeks) and the MN assay was repeated at the end of week 6. In subgroup B (RA-MTX-MTX/Folic Acid) the MN assay was repeated 6 weeks later, and then the patients were placed on folic acid (5 mg per day for 6 weeks) and the MN assay was repeated at the end of this period.

The number of micronuclei in RA patients was compared with a reference (control) group from the Mutagenesis Laboratory of the same hospital. This group consisted of 39 healthy individuals who were taking no medication and who had no history of smoking or alcohol consumption. Twenty-six (67%) were females and the mean age of this group was 34 ± 12 years (range 21-68 years).

MN sample preparation

All patients were asked to rinse their mouths with water before the mucosal sweeps. A polished slide was used to collect cells from the oral mucosa of the right and left cheeks, then the samples were spread directly in two separate slides. The smears were air dried and fixed in 80% methanol for 48 h and stained using Orcein as follows. After immersion in 1 N HCl at 60°C for 8 min, the slides were immersed in Orcein reagent for 20 min, rinsed with distilled water, Fast Green contrasted for 30 seconds and rinsed with distilled water. After this procedure the nucleus was stained in pink or orange and the cytoplasm in blue (Fig. 1).

The slides were analyzed by one of two experienced technicians using a light microscope (100 x); the technicians were blinded to the patient data group assignments. Each reader counted the number of micronuclei in a field of 2000 cells per slide (15). The guidelines for identifying MN in oral epithelial cells was based on their morphology and location within the cell, as previously described (16). Briefly, morphology criteria included texture comparable to the main nucleus, round to oval shape with a distinct boundary, and size less than one-third the diameter of the main nucleus.

Statistical analysis

Differences between 2 continuous variables were compared with 2-tailed t tests. Differences between 3 or more continuous variables were compared with one way analysis of variance, using Scheffe's method for multiple comparisons. Differences between proportions were compared with the chi-squared test and Fisher's exact test. Statistical significance was set at $p \leq 0.05$, and the confidence interval at 95%. Inter-observer variability was assessed using Pearson's correlation in 25 randomly selected slices read by 2 technicians who were blinded to the patient data, assignation or previously detected number of MN. The study was approved by the Ethics Committee of the Hospital de Especialidades.

Results

The demographic and clinical characteristics of RA patients are shown in Table I. There were 50 RA patients taking MTX and 30 RA patients who had never used MTX or azathioprine and who were on other second line drugs, mainly chloroquine (73%). Most pa-

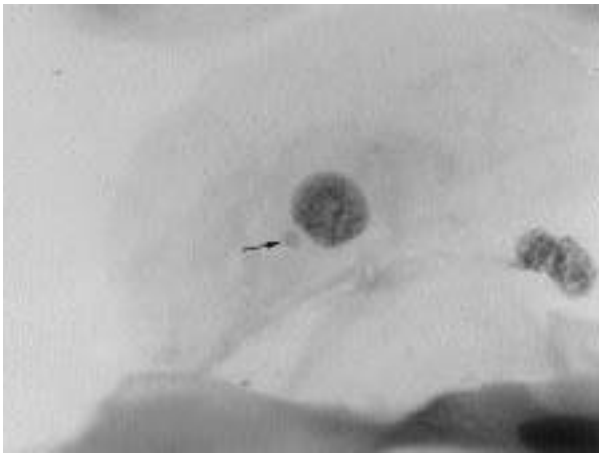

Fig. 1. Bucal mucosal cell stained with Orcein and fast green showing a micronuclei (arrow) (100x).

Table I. Demographic and clinical data of the 80 RA patients.

Variables	Rheumatoid Arthritis Using MTX n = 50	Not using MTX n = 30	P value
Age, mean yrs ± SD	46 ± 10	48 ± 14	NS
Education, mean yrs ± SD	5 ± 3	7 ± 4	0.018
Gender, women, n (%)	40 (80)	26 (87)	NS
Smoking, n (%)	13 (26)	1 (3)	0.001
Anticonceptive use, n(%)	6 (12)	3 (10)	NS
Cafein use, n (%)	19 (38)	4 (13)	0.018
RA duration, mean yrs. ± SD	12 ± 9	13 ± 9	NS
Prednisone use, n (%)	15 (30)	0	< 0.001
MTX duration, mean months ± SD	16 ± 18	-	-
MTX dose, mean mg per week ± SD	8.7 ± 1.5	-	-

Table II. Baseline differences in the number of MN in RA patients and controls.

	RA patients Using MTX n = 50	Not using MTX n = 29	Healthy Controls n = 39	P Value
MN, mean ± SD	3.14 ± 3.05	3.48 ± 1.54	0.8 ± 0.8	< 0.001*
MN, 95% CI	2.27 to 4.0	2.89 to 4.07	0.53 - 1.07	

* Significant differences between RA patients and controls, but not between users and non-users of methotrexate.

Table III. Frequency distribution of micronuclei number among study groups.

MN by range	RA patients Using MTX n = 50	Not using MTX n = 29	Controls n = 39
Normal (0 - 2), n (%)	29 (58)	10 (35)	37 (95)
Moderate (3 - 5), n(%)	14 (28)	6 (55)	2 (5)
High (> 6), n (%)	7 (14)*	3 (10) *	0 (0)*

* p< 0.001 between RA patients and controls, but not between users and non-users of methotrexate.

tients were female. The mean age (range 20 - 76 years) and mean disease duration (range 1 to 39 years) did not differ between the two groups. However, the MTX group was slightly less educated and had a higher proportion of smokers, coffee drinkers and prednisone users. Methotrexate was being used p.o. in all cases for a mean period of 16 months (range 1-78), a mean dose of 8.7 mg, and a range of 7.5-12.5 mg/wk.

The interobserver correlation of the MN assay was 0.82. Table II shows the differences in MN between both groups of RA patients and the healthy controls. The MN test from one RA patient in the non-MTX group could not be assessed due to the poor quality of the slide. At baseline the number of MN was significantly higher in the RA patients than the controls ($p < 0.001$, 95% C.I. for differences from 1.2 to 3.5 for MTX users, and 1.3 to 4 for non-users of MTX). However, no difference in the MN number was observed between users and non-users of MTX.

Table III shows the frequency distribution of MN among the study groups. Values of MN were defined as normal when between 0 and 1 SD of the controls (0 to 2), as moderately increased when between 3 and 5, and high when MN were ≥ 6. MN in RA patients were more frequently in the moderate to high range compared with controls, whether or not they were users of MTX ($p < 0.001$).

We observed no effect of smoking on the MN number. Cigarette consumption was documented in 14 patients (13 out of 50 in the MTX-RA group, and 1 out of 30 in the non-MTX RA group). Four of these were heavy smokers: one smoked an average of 40 cigarettes per day, and 3 an average of 20 per day. The MN number in these 4 cases was zero. The other 10 patients smoked less than 6 cigarettes per day and the MN number showed no differences compared with the controls. No significant associations were found between the MN number and other variables, including caffeine consumption, contraceptive use, methotrexate dosage or duration, and RA duration.

The effect of folic acid supplementation on the MN number was assessed in 40 out of 50 MTX treated RA patients; the remaining 10 (20%) patients were lost to follow-up. No significant differences were found between the mean MN number at baseline and after folic acid supplementation (MN mean 3.08 ± 2.9 vs 3.1 ± 2.8, $p = 0.94$, 95% CI for differences -1.4 to 1.34).

Discussion

It seems that the distribution of site-specific cancers in RA is skewed compared with the general population; the incidence of lymphomas and bladder cancer is increased while the incidence of cancer of the digestive organs is decreased (1, 2). This could be explained by the influence of the disease itself and the drug treatment that is used in RA patients. However, there are some problems in ascertaining the influence of specific DMARDs on cancer induction. The first is the long latency period between exposure to a DMARD and the development of malignancy. The second is that RA patients frequently switch from one DMARD to another, or use a combination of DMARDs during the course of their disease.

The MN test permits the assessment in short-term studies of the genotoxicity associated with RA and/or the drugs used to treat it. The detection of MN is widely used as a short-term mutagenicity test because it is a simple and rapid screening technique of chromosomal damage in cytological preparations, compared with assays based on the detection of chromosomal aberrations and sister chromatid exchange. The MN test has been applied in the biological monitoring of human populations exposed to mutagenic and carcinogenic agents. It is a promising tool for the study of carcinogens in exfoliated cells. The exfoliated cell MN assay has advantages over the more widely used MN test in lymphocytes. It is a non-invasive technique and repeated sampling is acceptable (7). Also, while lymphocytes

must be stimulated to undergo mitosis, epithelial cells do not need to be stimulated. MN in exfoliated cells reflect genotoxic events that occurred in the dividing basal cell layer 1 to 3 weeks earlier (7).

There are four recognized mechanisms by which micronuclei and micronucleus-like structures can arise: (i) mitotic loss of eccentric fragments; (ii) a variety of mechanical consequences of chromosomal breakage and exchange; (iii) mitotic loss of whole chromosomes, and (iv) apoptosis (17). However, as exfoliated cells frequently undergo morphologic changes leading to cell death and nuclear disruption, it is important to distinguish between degenerative nuclear phenomena and true micronuclei. The MN readings performed in this study are reliable and reproducible. This affirmation is sustained by three points that should be followed in similar studies: a) the technicians must identify MN following previously reported guidelines (16),b) cells undergoing any kind of degeneration (karyolysis, karyorrhexis, pyknosis and nuclear fragmentation) must be excluded from evaluation, and c) the reference (control) group used should have a mean MN similar to the healthy controls in other studies. In our study, healthy controls had a mean of 0.8 MN, which is quite similar to the reported means in healthy controls from other studies (7, 8, 19).

It has been shown that MTX induces MN in mouse bone marrow cells (11) and mice peripheral blood reticulocytes (12). It has also been reported in an *in vitro* study that folinic acid inhibited the induction of MN by MTX (13). We found two studies assessing MN in RA patients. In the first study, Jensen *et al.* did not find an increased number of MN in bone marrow cells from 11 patients with RA treated with penicillamine (18). In the other study, Prosser *et al.* did not find a significant increase in MN in peripheral lymphocytes prior to and 2 weeks after radiation synovectomy in patients with RA or osteoarthritis of the knee (19). However, to the best of our knowledge MN induction has not been studied in MTX treated RA patients.

In this study two different groups of RA patients were assembled. The first group consisted of RA patients taking MTX in different doses and for different durations. The second group consisted of RA patients who had never used MTX. MN test were performed in all of these patients and the results were compared with healthy controls. These groups allowed an assessment of whether RA patients who were or were not using MTX have more MN than healthy controls.

The third aim of this study was to assess if folic acid supplementation decreased the number of MN in MTX treated RA patients, as was suggested by an experimental study (13). To accomplish this we used a randomized, cross-over design. Ten patients (20%) out of the expected 50 patients on folic acid supplementation were lost to follow-up. We found that the MN number did not decrease after folic acid supplementation, at least at the doses and duration used in this study.

In this study we found that the mean MN was 3-fold higher in RA patients compared with controls, regardless of their MTX use. This suggests that RA itself rather than MTX induces MN, which is in accord with the suggestion that the increased risk of leukemia may be due to RA itself since other potential explanatory factors for leukemia, such as the use of DMARDs, were not apparent in a cohort study (1). However, our suggestion that RA itself induces MN should be viewed with caution since we did not analyse certain other factors, such as Sjögren's syndrome, chloroquine and NSAIDs use, which might also be associated with MN induction. Indeed, MN induction by higher doses of MTX than those used in our study cannot be excluded. To the best of our knowledge, chloroquine-associated MN induction has not been assessed in RA patients; experimental and *in vivo* studies, however, some previous results suggests that this drug does not induce MN formation (20). Tobacco, mainly in its chewing formulation, increases the number of MN (16). We found no effect of smoking on MN induction; this could be explained by the low number of cases and the low daily cigarette consumption in most of the patients. None of our patients used tobacco in other formulations.

In summary, our results suggest that genotoxicity is present in RA and that it is associated with the disease itself rather than MTX use. Further studies should confirm our results and determine the influence of other drugs and Sjögren's syndrome on the induction of MN. The value of MN as a predictor of cancer remains to be elucidated.

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