The Induction of Apoptosis by Methotrexate in Activated Lymphocytes as Indicated by Fluorescence Hyperpolarization: a Possible Model for Predicting Methotrexate Therapy for Rheumatoid Arthritis Patients

Shoshy Herman¹, Naomi Zurgil¹, Pnina Langevitz², Michael Ehrenfeld², and Mordechai Deutsch^{1*}

¹The Biophysical Interdisciplinary Schottenstein Center for the Research and the Technology of the Cellome, Physics Department, Bar-Ilan University, Ramat-Gan 52900 Israel and ²Tel Hashomer, Department of Rheumatology, Sheba Medical Center, Tel Hashomer, Israel

ABSTRACT. The objectives of this study were to test the *in vitro* response of healthy non-activated, activated, and rheumatoid arthritis (RA) lymphocytes to methotrexate (MTX), and design an *in vitro* model for predicting the efficiency of MTX treatment for RA patients. Considering the RA profile of clonal-expanded CD4⁺ T cells, phytohemagglutinin-activated mononuclear cells taken from healthy donors were incubated with different concentrations of MTX. The MTX-immunosuppressive effect was tested by fluorescence intensity measurements, including PI assay and annexin V assay. For simple detection, we used the Individual Cell Scanner (IC-S), which enables the measurement of early events in individual cells. Healthy mononuclear cells (MNC), and MNC derived from RA patients, were tested by the IC-S while utilizing fluorescence polarization (FP) measurements of fluorescein diacetate (FDA) as an established marker of activation or suppression. In healthy activated MNC, we found that MTX, through its early incubation period, interferes with the activation signal obtained by PHA and exerts an apoptotic signal, which is noted by increases in the FP. Comparing our model to six long-standing RA patients and five newly-diagnosed patients revealed significant differences in the FP measurements, including fluorescence depolarization as an early established measurement of lymphocyte activation, and hyperpolarization as a measurement of an early immunosuppressive effect. We conclude that MTX, an effective therapy for RA patients, could easily be tested by fluorescence polarization measurements of FDA before (or during) clinical use in order to predict its efficiency on a specific RA patient. Moreover, the FP measurements can be used for the diagnosis, and making timing and dosage decisions.

Key words: Rheumatoid Arthritis (RA)/Methotrexate (MTX)/Individual Cell Scanner (IC-S)/Mononuclear Cells (MNC)/Fluorescence Polarization (FP)

Methotrexate (MTX), a systemic chemotherapeutic agent originally used for malignant disorders (Jackson *et al.*, 1984), is presently being used to treat inflammatory disorders as well (Willkens *et al.*, 1984). MTX was found to be the preferred drug for patients suffering from rheumatoid

arthritis (RA) (Weinblatt *et al.*, 1985; Wolfe *et al.*, 1990). While in chemotherapy MTX doses are definitely high (up to 30 $\mu\text{g}/\text{m}^2$), in autoimmune diseases a low MTX dose of 7–15 mg/week was found to be effective.

It has been realized over the past decade that early diagnosis and early treatment are critical for RA patients. Moreover, an overall plateau of clinical response to MTX is reached after only six months of treatment. Therefore, enabling patients with early arthritis to access the appropriate therapy is of great importance. Additionally, because the last decade has seen many RA patients who failed to respond to the MTX drug, the availability of new options could bring more optimal care to them. Therefore, we find it necessary to predict the immunosuppressive effects of MTX.

*To whom correspondence should be addressed: Mordechai Deutsch, Ph.D., The Biophysical Interdisciplinary Center for the Research and the Technology of the Cellome and The Schottenstein Center for Early Detection of Cancer, Physics Department, Bar-Ilan University, Ramat-Gan 52900, ISRAEL.

Tel: +972–3–5344675, 5318349, 5354148, Fax: +972–3–5342019

E-mail: rtcellom@mail.biu.ac.il

Abbreviations: RA, rheumatoid arthritis; MTX, methotrexate; IC-S, Individual Cell Scanner; MNC, mononuclear cells; FDA, fluorescein diacetate; FP, fluorescence polarization; PHA, phytohaemagglutinin; PI, propidium iodide; AICARibotide, 5-amino-4-imidazolecarboxamide-riboside-5'-monophosphate.

So far, the following findings have been cited to explain MTX's immunosuppressive and anti-inflammatory properties at the cellular level:

a) MTX inhibits folate-dependent enzymes via polyglutamated derivatives, which leads to anti-proliferation (Chabner *et al.*, 1985; Sperling *et al.*, 1990; Fairbanks *et al.*, 1999). The inhibition by MTX of the folate dependent enzyme 5-amino-4-imidazolecarboxamide-ribose-5'-monophosphate (AICARibotide) transformylase can lead to the intracellular accumulation of AICARibotide, as demonstrated with an *in vivo* model of inflammation (Cronstein *et al.*, 1993).

b) MTX mediates the release of adenosine, which suppresses inflammation (Cronstein *et al.*, 1991). Subsequent studies implicated adenine nucleotides, released as a result of cellular injury or necrosis, as being the source of the extracellular adenosine, in which AICARibotide might be involved indirectly (Morabito *et al.*, 1998).

c) Recently, it was proposed by Genestier *et al.* that the immunosuppressive properties of MTX relate to the induction of apoptosis, especially in activated T cells (Genestier *et al.*, 1998; Nishioka *et al.*, 1998).

Surprisingly, MTX in normal *in vitro* mononuclear cells has received little attention. Genestier *et al.* were the first to demonstrate that apoptosis of activated, healthy T-cells by MTX does occur, and probably does not require the interaction between CD95 (FAS, APO-1) and its ligand. In addition, adenosine release by these cells could account for only a small part of MTX activity (Genestier *et al.*, 1998). An additional single study concentrates on MTX suppressive effect in mitogen stimulated healthy T-lymphocytes, and suggests that MTX inhibits the first committed step of purine biosynthesis (Fairbanks *et al.*, 1999). Both of the latter reports suggest that apoptosis by MTX requires progression of activated T-cells to the S-phase of the cell cycle, as evidenced by drug or antibody interference with IL-2 synthesis or signaling pathway.

Considerable evidence suggests that the cell subpopulation T-helper 1 (Th1) plays a predominant role in RA pathogenesis. Lymphocytes obtained from RA patients produce Th1 type cytokines (Singh *et al.*, 1999; Kusaba *et al.*, 1998). Cytokines produced by Th cells are of critical importance for the disease development or subsiding (Infante-Durate and Kamradt, 1999). Rudwaleit *et al.* report on *in vivo* immunosuppressive effect of MTX, manifested by a decrease in TNF α production and an increase in IL-10 production (Rudwaleit *et al.*, 2000), suggesting MTX involvement in a cellular regulatory mechanism relevant to the disease remission. Another study evaluates the influence of long term therapy by MTX on the general normalization of the cytokine balance (Th1/Th2) in both CD4⁺ and CD8⁺ lymphocytes (Schuerwegh *et al.*, 2001).

The clonal expansion of CD4⁺ T cells is a characteristic finding in patients with RA (Goronzy *et al.*, 1994). Expanded CD4⁺ clones are present in the blood and in-

filtrate into the joint, persisting for years (Waase *et al.*, 1996; Schmidt *et al.*, 1996). Based on the latter report (Genestier *et al.*, 1998) and our earlier findings, which indicated that lymphocyte activation as well as apoptosis can be monitored and measured by fluorescence polarization (FP) of FDA-labeled cells using the Individual Cell Scanner (IC-S) (Zurgil *et al.*, 2000; Zurgil *et al.*, 1999a; Deutsch *et al.*, 2000a), we designed this study to test the effects of MTX on activated lymphocytes.

Fluorescence polarization is considered to be one of the first functional cytometric parameters (Shapiro, 1995). It was initially suggested by the Cerceks as an indicator of lymphocyte activation following the incubation of the lymphocytes with phytohemagglutinin (PHA) (Cercek and Cercek, 1977), a finding that was later verified by others as well (Kaplan *et al.*, 1997; Orjasaeter *et al.*, 1979; Schnuda, 1980; Pritchard *et al.*, 1982; Rolland *et al.*, 1984; Sakamoto *et al.*, 1981).

In the present study, an *in vitro* model was designed to find the most suitable suppression effect of MTX on activated lymphocytes. The IC-S was used to monitor the development of the apoptotic process, following MTX administration, in an effort to establish an early event that demonstrates the MTX suppression effect. This model may help RA patients to receive the appropriate treatment, as well as to predict dosage and timing for better disease therapy.

Materials and Methods

Materials

Propidium iodide (PI), MTX, RNase, and trypan blue (TB) were obtained from Sigma (St. Louis, MO, USA). Fluorescein diacetate (FDA) was obtained from Riedle de Haen AG (Seize, Germany). Working dilutions (1.2 μ M in phosphate buffered saline (PBS)) were prepared from a 25 mM stock solution in acetic acid (Fruitarom, Israel). Ficoll-Paque was obtained from Pharmacia LKB, Sweden. Dulbecco's PBS containing 1.3 mM Ca²⁺ and 0.5mM Mg²⁺ and RPMI 1640 were obtained from Biological Industries (Beit Haemek, Israel). Phytohaemagglutinin (PHA, Wellcome, UK) was dissolved in PBS (0.9 mg/ml) and stored at -20°C.

Cell preparation

In this study were included 11 RA patients and 18 healthy volunteers. The RA patients were diagnosed according to the ACR criteria (Arnett *et al.*, 1988). The RA patient group included 6 RA patients (2 males and 4 females) treated by weekly oral administration of MTX (10 mg in 2 patients and 12.5 mg in 4 patients), and 5 recently diagnosed untreated RA patients (1 male and 4 females). The average age of the patients was 61 (ranging 34–81). All healthy volunteers had no history of any chronic disorders. The research was certified and conducted at the Sheba Medical Center, Tel Hashomer, Israel, under the Helsinki regulations. The study

participants gave their informed consent to donate 10 ml blood for examination.

Mononuclear cells (MNC) were isolated from healthy and RA peripheral blood using the Ficoll Paque procedure, as previously described (Kaplan *et al.*, 1997). Following separation, the cells were washed, twice in saline and once in PBS, and then suspended in enriched RPMI at a concentration of 1.5×10^6 cells/ml. Viability was checked by analyzing the ability to exclude TB by light microscope. PHA stimulation of MNC was performed by adding a final optimal concentration of 5 $\mu\text{g/ml}$ PHA to MNC for different incubation periods and incubating at 37°C , 5% CO_2 .

PI assay

Following treatment, 1.5×10^6 cells/ml were washed with PBS and stained with PI at a final concentration of 1 $\mu\text{g/ml}$. Fluorescent cells were counted under a fluorescence microscope.

Annexin V assay

One million cells were washed with PBS and resuspended in binding buffer (Genzyme, Cambridge, MA). FITC-annexin V was added to a final concentration of 1 $\mu\text{g/ml}$, while PI was simultaneously introduced. After 10 min of incubation in the dark at room temperature, the cells were analyzed by flow cytometry (FACScan BD).

Determination of apoptosis by measuring cell cycle status

In order to assess cell cycle and apoptosis, 1×10^6 cells were resuspended with 70% cold ethanol. Cells were settled by centrifugation and resuspended with 1 ml lysing buffer containing 40 $\mu\text{g/ml}$ RNase A. They were then stained with 100 $\mu\text{g/ml}$ PI and stored in the dark at 4°C for at least 30 min before FACS analysis. A total of 20,000 events were acquired with a FACScan (Becton Dickinson). Gates for apoptotic cells appeared to the left of the G_0/G_1 peak.

FDA staining

Treated or untreated cells were washed 3 times with incomplete RPMI 1640 medium, which lacked phenol red but contained 10 μM HEPES buffer solution. An aliquot of 50 μl of cell suspension (1.5×10^6 ml) was added to 50 μl FDA staining solution in PBS and incubated at room temperature for 5 min. At the end of incubation, the cells were settled in their traps on the cell carrier, as previously described (Deutsch and Weinreb, 1994), washed 3 times with fresh buffer in order to remove excess staining solution, and measured for fluorescence polarization (FP). The FP is defined as the ratio $(\text{FI}_{\parallel} - \text{FI}_{\perp})/(\text{FI}_{\parallel} + \text{FI}_{\perp})$, where (II) and (+) correspond to the FI measured parallel and perpendicular to the excitation field vector, respectively. The more the molecule rotational movement is restricted, the higher the FP value and vice versa. The FP is dependent on intrinsic molecular variables such as fluorescence lifetime and the moment of inertia, as well as on micro-environmental parameters of the media hosting the fluorophore probe, including viscosity and temperature.

FP is a powerful and sensitive technique for the study of macromolecular interactions, conformational changes, membrane fluidity, and cytoplasmic viscosity (Zurgil *et al.*, 1999b; Gidwani *et al.*, 2001; Dandliker and Feigen, 1961; Levison *et al.*, 1976; Jolley, 1996). The IC-S is a multiparametric multilaser-based static cytometer. It enables precise repeated measurements on selected individual cells within a population under changeable physiological conditions. On-line reagent addition or changes in the experimental conditions (i.e., addition of apoptosis inducers or inhibitors, a change of buffers) can be accomplished, and dynamic changes on given individual cells are followed in real time. Principal approaches of the IC-S methodology were published elsewhere (Deutsch and Weinreb, 1994).

IC-S apparatus

The IC-S apparatus was previously described in detail (Deutsch *et al.*, 2000a; Sunray *et al.*, 1999). In short, it is a multiparametric, computerized, discrete static cytometer, which was designed, built, and upgraded in our labs. Its central feature is the cell tray (CT), a microsieve incorporating a two-dimensional trap array with 20 μm pitched holes, each having an effective opening diameter of 7 μm and about 8 μm depth, in which individual cells are placed. The CT is mounted on a computer-controlled stage that enables repeated multi-scanning of the same cells.

In the present study, cells were irradiated with 1–10 μW of 442 nm light from a He-Cd laser. Under the staining conditions used here, the sampling time for obtaining a count of 10,000 photons from each dye-loaded cell varied from 0.001 sec to approximately 0.5 sec.

The acquired data, including cell position, measurement duration for each cell, absolute time, intensity at two different wavelengths, computed polarization values, and test set-up information, are displayed on the screen, on-line, graphically and numerically, and stored in the memory. Software enables the determination of the range and other statistical characteristics of all parameters, either for the entire cell population or for an operator-selected sub-population before or during the scan.

Statistical analysis

Data, expressed as mean $\pm$ SD, was analyzed by a two-tailed paired Student's *t* test. Differences were determined to be significant for $P \leq 0.05$.

Results

MTX induces an apoptotic effect on mitogen-activated MNC after 72 h

In order to emulate the RA MNC picture and further investigate the best potential effect of MTX on activated human MNC, the cells were incubated with PHA (5 $\mu\text{g/ml}$) for 24, 48, 72, and 96 h and then suppressed by MTX for 24 h (Fig. 1a). MTX concentrations ranging from 0.01–10 μM were tested, reproducing the low-dose therapy currently given to

RA patients. The optimal doses were found to be 0.1 μM and 1 μM . Cell death was demonstrated through the PI exclusion assay and cell viability, by the exclusion of TB. A time-dependent effect of suppression was noted with all doses of MTX (Fig. 1a). However, 96 h of proliferation was

Fig. 1. (a) Percentage of cell death by MTX administration. MNC (1.5×10^6) stimulated with PHA ($5 \mu\text{g/ml}$) for 24, 48, and 72 h, followed by incubation with different MTX concentrations (0.01–10 μM) for an additional 24 h. Results represent mean $\pm$ SE of 3 different experiments. * = $P \leq 0.05$. (b) Unstimulated MNC for 24, 48, and 72 h, incubated with different MTX concentrations (0.01–10 μM) for an additional 24 h. Results represent mean $\pm$ SE of 3 different experiments.

not sufficient to exert a significant suppression effect by MTX (data not shown). A remarkably significant increase in apoptotic cells (37%) resulted from 72 h of PHA activation followed by suppression with 1 μM MTX, as compared to 20% cell death in the absence of MTX (Fig. 1a). Moreover, testing all the doses at all examined time periods in the absence of PHA pre-stimulation indicated that MTX alone barely enhanced the apoptotic rate (Fig. 1b). During all stimulation times, a progressive decrease in cell viability was evident, which reflects the nature of proliferated cells. The most significant decrease was noted at 72 h of PHA stimulation (Fig. 2). This concurs with data in which 72 h of PHA stimulation followed by 1 μM MTX incubation enhanced the percentage of cell death. Ten μM MTX was found to be toxic for cell cultures (Fig. 2).

These data indicate that MTX inhibits cell proliferation and induces effective cell death of highly activated lymphocytes. Alternatively, at short proliferation signals (24 h), MTX yields a low decrease of viable cell counts. In order to confirm our data for the apoptotic process, two other tests were executed parallel to the PI test: cell cycle analysis for the pre G_1 event and the annexin V assay. The results show a pattern similar to the one described above. Incidence of apoptosis was substantially greater after pre-incubation of MNC with PHA for 72 h followed by suppression with MTX for another 24 h. The cell counts at the pre G_1 state were found to be 21.7% (Fig. 3a), as compared to cells suppressed by MTX alone, which yielded a count of 7.26% (Fig. 3b). Cells incubated with PHA alone for 72 h yielded a count of 12.68% (Fig. 3c).

The annexin V assay is summarized in Table I. Early and

Fig. 2. The effect of MTX on cell viability. MNC (1.5×10^6) stimulated with PHA ($5 \mu\text{g/ml}$) for 72 h, followed by 24 h incubation with (0.01–10 μM) or without MTX. Results represent mean $\pm$ SE of 3 different experiments. * = $P \leq 0.05$.

Fig. 3. Determination of pre-G1 phase of cell cycle by "FACS" analysis. (a) MNC pre-incubated with PHA (5 µg/ml) for 72 h, followed by incubation with 1 µM MTX. (b) MNC incubated for 72 h without any stimuli, followed by incubation for 24 h with MTX (1 µM) alone. (c) MNC incubated for 96 h with PHA (5 µg/ml) alone.

late apoptosis were measured by double staining with PI and annexinV-Fitc. As seen, a significant count of ($8^{\text{ANN}}+17^{\text{ANN+PI}}$) was noted when MNC were pre-stimulated with PHA and then treated with MTX. Unstimulated MNC which were treated with MTX yielded an average percentage of events ($3.5^{\text{ANN}}+7^{\text{ANN+PI}}$), which was quite similar to PHA-stimulated cells which were not treated with MTX. Completely untreated MNC exhibited the lowest percentage of apoptotic events ($4^{\text{ANN}}+4^{\text{ANN+PI}}$).

The IC-S Cytometer as a tool for monitoring MNC stimulation and suppression by FP measurements

The IC-S apparatus was used to evaluate, assess, and build a new detecting and investigating model for inducing apoptosis in 72 h proliferated lymphocytes. It has previously been shown that lymphocyte activation involves early structural changes in cytoplasmic fluidity, which can easily be monitored by fluorescence depolarization of cells labeled with fluorescein diacetate (FDA) (Sunray *et al.*, 1999; Deutsch *et al.*, 2000b). The more restricted the fluorescent probe mobility, the higher its FP, which is indicated by probe size, hosting media viscosity, and binding. Further studies showed that the apoptotic event can be demonstrated by the increase in fluorescence polarization (FP) of FDA (Zurgil *et al.*, 2000). FDA FP measurements were used in this study to further analyze the *in vitro* response of activated lymphocytes to MTX.

Fig. 4 demonstrates a typical histogram distribution of FP values of FDA-labeled MNC that have been stimulated for 96 h with PHA, MTX, or a combination of both. As can be seen, unstimulated MNC, used as control cells (CON), yield a high mean FP value (0.187), while those treated with only PHA for 96 h exhibited the lowest mean value of FP. Interestingly, MNC treated with MTX alone during the last 24 h exhibited a pattern similar to that of untreated cells. Following treatment with PHA for 72 h and MTX during the last 24 h, an intermediate mean FP value was obtained, indicating the occurrence of apoptosis, which, as discussed above, is manifested by hyperpolarization. Table II, which summarizes various experiments similar to the typical histogram discussed above, confirms the above data where a depolarization signal is evident following 72 h of PHA activation of normal MNC and hyperpolarization, which is maintained by MTX involvement.

Since intracellular fluorescein hyperpolarization was found to be an indicator of early apoptotic events (Zurgil *et al.*, 2000), the next step was to trace early signals of the suppression of activation by MTX during its first hours of incubation via the IC-S. MTX (1 µM) was added to MNC that had been activated for 72 h with PHA and immediately tested for FDA FP. FDA FP was measured during the first 3

Table I. ANNEXIN V ASSAY

		% of events Annexin ^{POS}	% of events PI ^{POS}	% of events Annexin ^{POS} +PI ^{POS}	% of events Annexin ^{NEG} +PI ^{NEG}
1	Control	4±0.4	8±1.0	4±0.8	84±4.1
2	PHA (5 µg/ml)	3±0.5	13±1.2	8±0.3	76±3.1
3	MTX (1 µM)	3.5±0.2	10±0.2	7±0.9	79.5±5.4
4	PHA+MTX	8±0.9*	10±0.5	17±1.5*	65±3.2*

Distinction between treated and untreated healthy MNC with both PHA and MTX. Results represent mean±SE of three different experiments. *= $p \leq 0.05$.

Fig. 4. A scatter histogram of the FP of FDA-labeled cells. MNC pre-incubated both with and without PHA for 72 h, followed by suppression with (1 μ M) MTX for 24 h.

Table II. THE % CHANGE OF FP VALUES OF FDA

Treatment	FP	% of Change
Control	0.207 $\pm$ 0.005	0
PHA (5 μ g/ml)	0.185 $\pm$ 0.003*	11
MTX(1 μ M)	0.196 $\pm$ 0.007	5.5
PHA+MTX	0.191 $\pm$ 0.006*	8

The % change of FP values of FDA, measured using (1.5×10^6) cells stimulated either by PHA or MTX or by a combination of both. Results represent mean $\pm$ SE of three different experiments.

*= $p \leq 0.05$.

h of MTX addition. Fig. 5 shows that the hyperpolarization signal was evident and more significant during the second hour of MTX incubation (15%), as compared to only 6.5% during the third hour, with a continuous moderate decrease over 24 h of suppression.

Polarization measurements of MNC derived from RA patients

Finally, the question arose as to the capability of the IC-S to recognize the apoptotic signal triggered by MTX in MNC obtained from RA patients (via FDA FP measurements). For this purpose, six RA patients treated with MTX (10–12.5 mg/week) were tested. All of them exhibited low FP values indicating their activation status (Table III), a result similar to that obtained in normal PHA-activated lympho-

Fig. 5. Hyperpolarization time-dependency of PHA-activated cells treated with MTX. MNC (1.5×10^6) activated with PHA (5 μ g/ml) for 72 h, followed by simultaneous FDA staining and MTX administration.

cytes. *In vitro* exposure of RA lymphocytes to MTX (1 μ M) for 24 h yielded an increase in the FP values of the cells, meaning an apoptotic effect by MTX was noticed.

Five newly-diagnosed, but not yet treated, RA patients exhibited a high FP value (average FP of 0.202) (Table III), which was significantly depolarized in 4 of these patients by the *in vitro* addition of PHA and MTX. The polarization rate of the fifth patient remained the same, a pattern that will be

Table III. FP VALUES OF FDA

<i>In vitro</i> treatment for RA patients recently diagnosed	FP Values
Control	0.202±0.003
PHA+MTX	0.186±0.004*
PHA	0.190±0.002
MTX	0.199±0.004
<i>In vitro</i> treatment for RA patients clinically treated with MTX	
Control	0.187±0.009
MTX	0.198±0.03*

The % change of FP values of FDA, measured using (1.5×10^6) cells stimulated either by PHA or MTX or by a combination of both. Results represent mean±SE of three different experiments.

*=p≤0.05.

discussed further. The *in vitro* exposure of these MNC to PHA alone considerably decreased their FP value, meaning that the MNC were not fully activated. Notably, the *in vitro* addition of MTX alone did not affect the FP value.

Discussion

The present study demonstrates that *in vitro* treatment of proliferated healthy human mononuclear cells with MTX yields a significant apoptotic effect, a phenomenon that was first noticed by the Genestier group (Genestier *et al.*, 1998). These studies provide substantial evidence of the beneficial effects of a low weekly dose of MTX in RA patients (Morgan *et al.*, 1990; Leeb *et al.*, 1990) who are characterized by the hyperproliferation of CD4⁺ cells (Goronzy *et al.*, 1994; Waase *et al.*, 1996; Schmidt *et al.*, 1996). The mechanism by which MTX stimulates suppression in RA patients is still debated (Chabner *et al.*, 1985; Sperling *et al.*, 1990; Fairbanks *et al.*, 1999; Cronstein *et al.*, 1991; Segal *et al.*, 1989; Seitz *et al.*, 1996; Hooper *et al.*, 1999). In this study, we tried to establish an early change in fluorescence polarization mediated by MTX, which indicates an apoptotic effect and could serve as a simple model to predict MTX efficiency in a specific RA patient.

The PI exclusion test indicates that the more the healthy MNC are activated, the more noticeable is the suppression by MTX. In order to confirm the above observation, we checked our model with flow cytometry. We specifically identified the apoptotic cells at the pre-G1 phase of the cell cycle. Here again, the significant suppression effect of MTX was observed only when healthy MNC were activated by PHA for 72 h. The annexin V assay supports the above data in proving the fact that the apoptotic signal is more noticeable in activated lymphocytes suppressed by MTX. In the report by Genestier *et al.*, MTX had been shown to exert an

apoptotic effect after 8 h of exposure to MNC, which were then activated by different stimuli in a drug-free medium for 24 h (Genestier *et al.*, 1998). In our model, we contend that stimulation of lymphocytes should be first followed by suppression by MTX – a condition typically found in RA patients.

The present study reports that MTX induces, in a time-dependent manner, the best apoptotic effect at 72 h for hyperblasting lymphocytes, which have been morphologically viewed under a fluorescence microscope (data not shown). This hyperblasting formation of lymphocytes has been commonly reported to be one of the features of RA patients, at various stages of the disease (Harris, 1990; Stamenkovic *et al.*, 1988; Feigenbaum *et al.*, 1979).

The progressive increase in apoptotic cells after the prolonged incubation period (72 h) was noticeable mostly in MTX administration ranging from 0.1 to 1 μ M MTX. This model provides evidence for the immunosuppressive activity of low dose MTX, which is clinically effective in RA patients. Moreover, this *in vitro* model can serve as a tool for optimizing dosage and timing of low dose MTX administration for RA patients.

In order to advance and assess our model, we utilized our past experience with the advantages of the IC-S cytometer (Zurgil *et al.*, 2000; Zurgil *et al.*, 1999a; Deutsch *et al.*, 2000a; Sunray *et al.*, 1999; Deutsch *et al.*, 2000b). Previous experiments with individual lymphocytes enabled the identification of lymphocyte activation by FDA fluorescence depolarization, as well as identification of early apoptotic events by FDA hyperpolarization (Cercek *et al.*, 1978). Based on the above, we were interested in examining our model, where effective apoptosis is achieved for lengthy lymphocyte activation. Upon examining the apoptotic signals induced after 24 h of MTX administration, in conjunction with the previously described model, an intermediate FP value was revealed, reflecting the conflicting message(s) transported to the nuclei. FP levels reflect the rotational mobility level of the fluorescent molecule. FP measurement provides insight into the nature of macromolecular interactions, ligand binding, and conformational dynamics (Carrero *et al.*, 1996; Thevenin *et al.*, 1994), as well as intracellular protein processing (Weaver *et al.*, 1997).

MNC activated for 96 h with PHA alone yielded the previously described characteristic behavior of activated lymphocytes. MNC that were activated with PHA for 72 h and then exposed to 1 μ M MTX for 24 h yielded a hyperpolarization signal typical of the apoptotic process, but atypical for PI observations where MTX alone barely enhanced the pre-G1 phase. Since PI exclusion and FDA polarization tests reflect two different properties related to the apoptotic reaction, it is assumed that the two assays should be analyzed on the basis of the stages involved in the apoptotic process. Early changes involved in the apoptotic process include cell dehydration and shrinkage, both of which were reported as having been identified by fluorescein

hyperpolarization (Darzynkiewicz *et al.*, 1997). On the other hand, the commonly used PI assay monitors cells according to their DNA content, in addition to their size, and analyzes the late parameters of cell death (Cohen, 1993). Based on the above, the effect of MTX administration on activated MNC was examined during the first minutes and hours of stimulation via FP measurement. The change in FDA FP is most significant during the second hour of suppression with MTX, while the third hour of suppression revealed a smaller change in FP, which decreases with time and is evident up to 24 h (Zurgil *et al.*, 2000).

Final experiments using MNC derived from long-standing RA patients indicate that the IC-S apparatus recognizes disease progression and predicts MTX efficiency by measuring FP. All six RA patients who were clinically identified as progressive exhibited low FP values, which correlated with the activation signal that was artificially created in our model using PHA. Moreover, *in vitro* addition of MTX significantly increased their FP, a pattern which characterizes the apoptotic signal. We assume that the MTX dosage (10–12.5 mg/week) clinically given to these patients has not achieved its complete efficiency and a higher dose (12.5–25 mg/week) should be considered. Interestingly, clinical data obtained from these patients did not show any significant improvement (including the patient's overall evaluation and the number of tender and swollen joints) during the period of six months following the start of treatment.

FP values obtained from diagnosed but untreated RA patients yielded a high FP value similar to that of the normal MNC, described previously in the *in vitro* model. The further the disease progresses, the more activated the lymphocytes become, thereby enabling possible recognition by their low FP activation value. *In vitro* incubation of these RA lymphocytes with PHA followed by MTX significantly decreased the FP value in four of the five patients, indicating that MTX treatment could be the appropriate treatment for those four patients. The remaining patient should consider other therapeutic options. The MNC, treated *in vitro* by PHA alone, show a decrease in FP, indicative of their ability to be further activated. *In vitro* addition of MTX alone did not affect the MNC, a phenomenon explained by the effectiveness of MTX in activated lymphocytes. Another possible explanation could be MTX's increased effectiveness after prolonged treatment (Wolfe *et al.*, 1990; Weinblatt *et al.*, 1985).

This study may greatly benefit RA patients who are still undecided about pursuing MTX treatment. Our ability to trace and quickly predict MTX immunosuppression using our model should definitely be a great advantage for RA patients. In the future, we hope to focus on utilizing and adjusting this model for other DMARD (disease modifying anti-rheumatic drug) options to improve the treatment and provide optimal care for these patients.

Acknowledgments. This research was supported by the Horowitz Founda-

tion and the Yael Research Fund.

References

- Arnett, F.C., Edworthy, S.M., Bloch, D.A., McShane, D.J., Fries, J.F., Cooper, N.S., Healey, L.A., Kaplan, S.R., Liang, M.H., and Luthra, H.S. 1988. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum.*, **31**: 315–324.
- Carrero, J., Mallender, W.D., and Voss, E.W. Jr. 1996. Anti-metatype antibody stabilization of Fv 4-4-20 variable domain dynamics. *J. Biol. Chem.*, **271**: 11247–11252.
- Cercek, L. and Cercek, B. 1977. Application of the phenomenon of changes in the structuredness of cytoplasmic matrix (SCM) in the diagnosis of malignant disorders. *Europ J. Cancer*, **13**: 903–915.
- Cercek, L., Cercek, B., and Ockey, C.H. 1978. Fluorescein excitation and emission polarization spectra in living cells: changes during the cell cycle. *Biophys. J.*, **23**: 395–405.
- Chabner, P.A., Allegra, C.J., Curt, G.A., Clendeninn, N.J., Baram, J., Koisumi, S., Drake, J.C., and Joliver, J. 1985. Polyglutamation of methotrexate. Is methotrexate a prodrug? *J. Clin. Invest.*, **76**: 907–912.
- Cohen, J.J. 1993. Apoptosis. *Immunol. Today*, **14**: 126–130.
- Cronstein, B.N., Eberle, M.A., Gruber, H.E., and Levin, R.I. 1991. Methotrexate inhibits neutrophil function by stimulating adenosine release from connective tissue cells. *Proc. Natl. Acad. Sci. USA*, **88**: 2441–2445.
- Cronstein, B.N., Naime, D., and Ostad, E. 1993. The antiinflammatory mechanism of methotrexate. Increased adenosine release at inflamed sites diminishes leukocyte accumulation in an *in vivo* model of inflammation. *J. Clin. Invest.*, **92**: 2675–2682.
- Dandliker, W.B. and Feigen, G.A. 1961. Quantification of the antigen-antibody reaction by the polarization of fluorescence. *Biochem. Biophys. Res. Commun.*, **5**: 299–305.
- Darzynkiewicz, Z., Juan, G., Li, X., Gorczyca, W., Murakami, T., and Tragnos, F. 1997. Cytometry in cell necrobiology: analysis of apoptosis and accidental cell death (necrosis). *Cytometry*, **27**: 1–20.
- Deutsch, M. and Weinreb, A. 1994. Apparatus for high precision repetitive sequential optical measurement of living cells. *Cytometry*, **16**: 214–227.
- Deutsch, M., Zurgil, N., Kaufman, M., and Shapiro, H. 2000a. Analysis of enzyme kinetics in individual living cells utilizing fluorescence intensity and polarization measurements. *Cytometry*, **39**: 36–44.
- Deutsch, M., Zurgil, N., Kaufman, M., and Berke, G. 2000b. Fluorescence of polarization as an early measure of T-lymphocyte stimulation. In *Methods in Molecular Biology*, **134**, T Cell Protocols: Development and Activation (Kearse, K.P., ed.). Humana Press, Inc., Totowa, NJ, pp. 221–241.
- Fairbanks, L.D., Ruckemann, K., Qiu, Y., Hawrylowicz, C.M., Richards, D.F., Swaminathan, R., Kirschbaum, B., and Simmonds, H.A. 1999. Methotrexate inhibits the first committed step of purine biosynthesis in mitogen-stimulated human T-lymphocytes; a metabolic basis for efficacy in rheumatoid arthritis. *Biochem. J.*, **342**: 143–152.
- Feigenbaum, S.L., Masi, A.T., and Kaplan, S.B. 1979. Prognosis in rheumatoid arthritis. A longitudinal study of newly diagnosed younger adult patients. *Am. J. Med.*, **66**: 377–384.
- Genestier, L., Paillot, R., Fournel, S., Ferraro, C., Miossec, P., and Revillard, J.P. 1998. Immunosuppressive properties of methotrexate: apoptosis and clonal deletion of activated peripheral T cells. *J. Clin. Invest.*, **102**: 322–328.
- Gidwani, A., Holowka, D., and Baird, B. 2001. Fluorescence anisotropy measurements of lipid order in plasma membranes and lipid rafts from RBL-2H3 mast cells. *Biochemistry*, **40**: 12422–12429.
- Goronzy, J.J., Bazzanella, P., Hu, W., Jendro, M.C., Walser-Kuntz, D.R., and Weyand, C.M. 1994. Dominant clonotypes in the repertoire of peripheral CD4⁺ T cells in rheumatoid arthritis. *J. Clin. Invest.*, **94**:

- 2068–2076.
- Harris, E.D., Jr. 1990. Rheumatoid arthritis. Pathophysiology and implication for therapy. *N. Engl. J. Med.*, **322**: 1277–1289.
- Hooper, M., Kallas, E.G., Coffin, D., Campbell, D., Evans, T.G., and Looney, R.J. 1999. Cytomegalovirus seropositivity is associated with the expansion of CD4⁺ CD28⁻ and CD8⁺ CD28⁻ T cells in rheumatoid arthritis. *J. Rheumatol.*, **26**: 1452–1457.
- Infante-Duarte, C. and Kamradt, T. 1999. Th1/Th2 balance in infection. *Springer Seminars in Immunopathology*, **21**: 317–338.
- Jackson, R.C., Fry, D.W., Boritzki, T.J., Besserer, J.A., Leopold, W.R., Sloan, B.J., and Elsager, E.F. 1984. Biochemical pharmacology of the lipophilic antifolate, trimetrexate. *Adv. Enzyme Regul.*, **22**: 187–206.
- Jolley, M.E. 1996. Fluorescence polarization assays for the detection of proteases and their inhibitors. *J. Biomolecular Screening*, **1**: 33–38.
- Kaplan, M.R., Trubniykov, E., and Berke, G. 1997. Fluorescence depolarization as an early measure of lymphocyte stimulation. *J. Immunol. Meth.*, **201**: 15–24.
- Kusaba, M., Honda, T., Fukuda, T., and Oizumi, K. 1998. Analysis of type 1 and type 2 T cells in synovial fluid and peripheral blood of patients with rheumatoid arthritis. *J. Rheumatol.*, **25**: 1466–1471.
- Leeb, B., Ogris, E., Dunky, A., and Mutzik, O. 1990. Pharmacokinetics of methotrexate in rheumatoid arthritis and psoriatic arthritis. *Arthritis. Rheum.*, **30**: A121.
- Levison, S.A., Dandliker, W.B., Brawn, R.J., and Vanderlan, W.P. 1976. Fluorescence polarization immunoassay: theory and experimental method. *Endocrinology*, **99**: 1129–1143.
- Morabito, L., Montesinos, M.C., Schreiber, D.M., Balter, L., Thompson, L.F., Resta, R., Carlin, G., Huie, M.A., and Cronstein, B.N. 1998. Methotrexate and sulfasalazine promote adenosine release by a mechanism that requires ecto-5'-nucleotidase-mediated conversion of adenine nucleotides. *J. Clin. Invest.*, **101**: 295–300.
- Morgan, S.L., Baggott, J.E., Vaughn, W.H., Young, P.K., Austin, J.V., Krumdieck, C.L., and Alarcon, G.S. 1990. The effect of folic acid supplementation on the toxicity of low-dose methotrexate in patients with rheumatoid arthritis. *Arthritis. Rheum.*, **33**: 9–18.
- Nishioka, K., Hasunuma, T., Kato, T., Sumida, T., and Kobata, T. 1998. Apoptosis in rheumatoid arthritis: a novel pathway in the regulation of synovial tissue. *Arthritis Rheum.*, **41**: 1–9.
- Orjasaeter, H., Hordfald, G., and Svendsen, I. 1979. Response of T-lymphocytes to phytohaemagglutinin (PHA) and to cancer-tissue-associated antigens, measured by the intracellular fluorescence polarization techniques (SCM test). *Br. J. Cancer*, **40**: 628–633.
- Pritchard, J.A.V., Sutherland, W.J., Siddall, J.E., Bate, A.J., Kerby, I.J., Deeley, T.J., Griffith, G., Sinclair, R., Davies, B.H., Rimmer, A., and Webster, D.J.T. 1982. A clinical assessment of fluorescence polarization changes in lymphocytes stimulated by phytohaemagglutinin (PHA) in malignant and benign diseases. *Eur. J. Cancer Clin. Oncol.*, **18**: 651–659.
- Rolland, J.M., Nairn, R.C., Nind, A.P., and Pihl, E. 1984. Significance of lymphocyte fluorescence polarization changes after phytohaemagglutinin stimulation in cancer and non-cancer conditions. *JNCI*, **72**: 267–274.
- Rudwaleit, M., Yin, Z., Siegert, S., Grolms, M., Radbruch, A., Braun, J., and Sieper, J. 2000. Response to methotrexate in early rheumatoid arthritis is associated with a decrease of T cell derived tumour necrosis factor ϵ , increase of interleukin 10, and predicted by the initial concentration of interleukin 4. *Annals of the Rheumatic Diseases*, **59**: 311–314.
- Sakamoto, S., Maida, H., Yamasaki, K., Aikawa, S., and Hinuma, Y. 1981. Evaluation of cellular fluidity of peripheral blood lymphocytes from patients with urogenital cancers by fluorescence polarization technique: PHA response and prognosis. *Eur. J. Cancer Clin. Oncol.*, **17**: 1055–1115.
- Schmidt, D., Goronzy, J.J., and Weyand, C.M. 1996. CD4⁺ CD7⁻ CD28⁻ T cells are expanded in rheumatoid arthritis and are characterized by autoreactivity. *J. Clinical Invest.*, **97**: 2027–2037.
- Schnuda, N.D. 1980. Evaluation of fluorescence polarization of human blood lymphocytes (SCM test) in the diagnosis of cancer. *Cancer*, **46**: 1164–1173.
- Schuerwegh, A.G., van Offel, J.F., Bridts, C.H., Stevens, W.J., De Clerck, L.S. 2001. Influence of longterm therapy with methotrexate and low dose corticosteroids on type 1 and type 2 cytokine production in CD4⁺ and CD8⁺ T lymphocytes of patients with rheumatoid arthritis. *J. Rheum.*, **28**: 1793–1799.
- Segal, R., Mozes, E., Yaron, M., and Tartakovsky, B. 1989. The effects of methotrexate on the production and activity of interleukin-1. *Arthritis Rheum.*, **32**: 370–377.
- Seitz, M., Loetscher, P., Dewald, B., Towbin, H., Rordorf, C., Gallati, H., and Gerber, N. 1996. Interleukin 1 (IL-1) receptor antagonist, soluble tumor necrosis factor receptors IL-1 beta, and IL-8-markers of remission in rheumatoid arthritis during treatment with methotrexate. *J. Rheum.*, **23**: 1512–1516.
- Shapiro, H.M. *Practical Flow Cytometry*. 1995. 3rd ed., Wiley-Liss, New York.
- Singh, V.K., Mehrotra, S., and Agarwal, S.S. 1999. The paradigm of Th1 and Th2 cytokines. Its relevance to autoimmunity and allergy. *Immunologic Research*, **20**: 147–161.
- Sperling, A., Benimeno, I., Arsten, K.F., Anderson, R., Colbyn, I., and Weinblatt, M.E. 1990. Effects of methotrexate therapy on 5-lipoxygenase pathway metabolism of arachidonic acid in rheumatoid arthritis. *Arthritis. Rheum.*, **33**: 173.
- Stamenkovic, I., Stegagno, M., Wright, K.A., Krane, S.M., Amento, E.P., Colvin, R.B., Duquesnoy, R.J., and Kurnick, J.T. 1988. Clonal dominance among T-lymphocyte infiltrates in arthritis. *Proc. Natl. Acad. Sci. USA*, **85**: 1179–1183.
- Sunray, M., Kaufman, M., Zurgil, N., and Deutsch, M. 1999. The trace and subgrouping of lymphocyte activation by dynamic fluorescence intensity and polarization measurements. *Biochem. Biophys. Res. Comm.*, **261**: 712–719.
- Thevenin, B.J., Periasamy, N., Shohet, S.B., and Verkman, A.S. 1994. Segmental dynamics of the cytoplasmic domain of erythrocyte band 3 determined by time-resolved fluorescence anisotropy: sensitivity to pH and ligand binding. *Proc. Natl. Acad. Sci. USA*, **91**: 1741–1745.
- Waase, I., Kayser, C., Carlson, P.J., Goronzy, J.J., and Weyand, C.M. 1996. Oligoclonal T cell proliferation in patients with rheumatoid arthritis and their unaffected siblings. *Art. Rheum.*, **39**: 904–913.
- Weaver, D.J. Jr., Durack, G., and Voss, E.V., Jr. 1997. Analysis of the intracellular processing of proteins: application of fluorescence polarization and a novel fluorescent probe. *Cytometry*, **28**: 25–35.
- Weinblatt, M.E., Coblyn, J.S., Fox, D.A., Fraser, P.A., Holdsworth, D.E., Glass, D.N., and Trentham, D.E. 1985. Efficacy of low dose methotrexate in rheumatoid arthritis. *N. Engl. J. Med.*, **312**: 818–822.
- Willkens, R.F., Williams, H.J., Ward, J.R., et al. 1984. Randomized double blind placebo controlled trial of low dose pulse methotrexate in psoriatic arthritis. *Arthritis Rheum.*, **27**: 376–381.
- Wolfe, F., Hawley, D.J., and Cathey, M.A. 1990. Termination of slow acting antirheumatic therapy in rheumatoid arthritis: a 14-year prospective evaluation of 1017 consecutive starts. *J. Rheumatol.*, **17**: 994–1002.
- Zurgil, N., Gerbat, S., Langevitz, P., Tishler, M., Ehrenfeld, M., Kaufman, M., Deutsch, M., and Shoenfeld, Y. 1999a. Detection of cellular activity in autoimmune disorders by the Cellscan system. In *The Decade of Autoimmunity* (Shoenfeld H. ed.). Elsevier Science, B.V., pp. 295–304.
- Zurgil, N., Kaufman, M., Solodiev, I., and Deutsch, M. 1999b. Determination of cellular thiol levels in individual viable lymphocytes by means of fluorescence intensity and polarization. *J. Immunol. Methods*, **229**: 23–34.
- Zurgil, N., Schiffer, Z., Shafran, Y., Kaufman, M., and Deutsch, M. 2000.

Fluorescein fluorescence hyperpolarization as an early kinetic measure of the apoptotic process. *Biochem. Biophys. Res. Comm.*, **268**: 155–163.

(Received for publication, November 21, 2002

and accepted, February 19, 2003)