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Mitotic index and cell proliferation kinetics for identification of antineoplastic activity

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The mitotic index (MI) and cell proliferation kinetics (CPK) of human blood lymphocyte cultures were determined to evaluate the effects of six antineoplastic drugs with well known cytostatic activity: cisplatin, melphalan, bleomycin, methotrexate, 5-fluorouracil and 6-mercaptopurine. All six drugs showed a clear effect on the inhibition of MI. The first three drugs interact directly with DNA showing a dose-related retardation of CPK. Methotrexate, 5-fluorouracil and 6-mercaptopurine, which act on ribonucleotide biosynthesis, showed no significant effects on CPK. The results suggest that CPK and MI measurements are useful for the prescreening of drugs with potential cytostatic activity.

Key words: Anti-cancer activity, cell proliferation kinetics, human lymphocytes, mitotic index.

Introduction

The study of antitumor activity of new drugs involves the use of a variety of tests, either *in vivo* or *in vitro*, which should select those compounds with anticancer potential.

The *in vitro* methods evaluate either cytotoxicity through dye exclusion, chromium release, incorporation of radiative DNA precursors[1,2] or the human tumor clonogenic assay (HTCA).[3] Some of the disadvantages of the first approach are that cytotoxicity is a very sensitive parameter yielding a large number of false positive results,[4] while in the HTCA not all tumor cell types grow well in culture and leukemias and lymphomas do not grow at all.[5,6] In addition, the HTCA is not sensitive to the effects of six of the most commonly used anticancer drugs.[7]

The use of mitotic index (MI) and cell proliferation kinetics (CPK) of lymphocyte cultures has been proposed as parameters for the screening of antineoplastic activity.[8] The CPK is a measure of the relative number of generations during a specified time in culture by the use of bromodeoxyuridine (BrdUrd);[9–11] this method identifies cells which have performed different numbers of DNA replications in culture.[11]

In the validation of a new biological system proposal for the screening of a specific activity, three points have to be taken into account: reproducibility, specificity and sensitivity of the parameters evaluated. The reproducibility of both MI and CPK has been reported in a previous work.[12] In this paper we evaluate the effects of six drugs with well known anticancer activity in order to determine the sensitivity of these endpoints for the detection of antineoplastic agents.

Materials and methods

Donors

Blood from five healthy donors (three women and two men with a mean age of 31 years) was obtained. We used blood from two donors in duplicate experiments to test the effects of each drug.

Lymphocyte cultures

Cultures were processed as described.[13] Briefly, 0.5 ml of heparinized blood was used to start cultures with RPMI 1640 culture medium, 32.5 μM of BrdUrd and 0.2 ml of phytohemagglutinin (PHA; Microlab, México); the total volume of the culture was ~ ml. The time of culture was ~2 h and the tested drugs were added 24 h before fixation. After fixation, cells were dropped onto slides and stained according to the fluorescence plus Giemsa method.[14]

Microscopic analysis was performed to determine the MI, scoring the number of metaphases in 2000 cells. The relative mitotic index (RMI) was evaluated as RMI = [MI treated MI control] × 100.

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The inhibition of mitotic index was calculated as 100 − [MI treated × 100/MI control]. CPK was evaluated on 100 consecutive metaphases determining the number of first (M1), second (M2) and third or further (M3) division cycles. The replication index (RI) was determined by the formula RI = M1 + 2(M2) + 3(M3)/100.

Chemicals

The following drugs were tested: cisplatin (Platinol; Bristol, Mexico; CAS 148-82-3), methotrexate (Sigma, St Louis, MO; CAS 59-05-2), bleomycin (Mack, Germany; CAS 11056-86-7), 5-fluorouracil (Sigma; CAS 51-21-8), 6-mercaptopurine (Sigma; CAS 50-44-2), melphalan (Sigma; CAS 148-82-3).

Statistical analysis

Statistical analysis for differences between donors was performed by a chi-square test, and a *t*-test was used for differences among concentrations and with respect to controls.[15]

Results

All drugs used induced a significant inhibition of the MI. With the exception of methotrexate the inhibition of MI was dose related. The effect on MI was larger in cells treated with bleomycin, methotrexate, melphalan, 5-fluorouracil and cisplatin than in those where 6-mercaptopurine was added (Figure 1).

Data also show that lymphocytes treated with different doses of cisplatin, melphalan and bleomycin exhibited a CPK retardation (Figure 2), while in cultures treated with 5-fluorouracil, 6-mercaptopurine and methotrexate no effect on CPK was detected (Figure 3).

Figure 4 shows the correlation between the percentage of inhibition of MI and RI. Drugs which interact with the DNA exhibited a dose curve with a negative slope, while 5-fluorouracil, methotrexate and 6-mercaptopurine (which do not act directly with DNA), showed slopes tending to zero.

Discussion

Preclinical screening of synthetic compounds or natural products for evidence of antitumor activity generally relies on evaluating only a single parameter, usually cell death. However, with the use of two biological endpoints, such as the MI and the CPK assays, more accurate information can be obtained. For instance, an inhibition of the MI can be interpreted in terms of death or as the arrest of cells at any moment during interphase.[16] On the

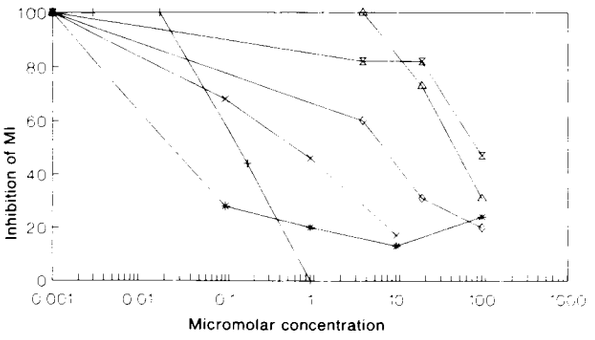

Figure 1. Effects of six antineoplastic drugs on the mitotic index (MI) of cultured human lymphocytes. Data are presented as percentage of inhibition of the MI. Results from untreated cultures are taken as 100%. Each point represents the mean value of two independent experiments. Drugs tested were: bleomycin (+), methotrexate (*), melphalan (×), 5-fluorouracil (◇), cisplatin (△) and 6-mercaptopurine (⧖).

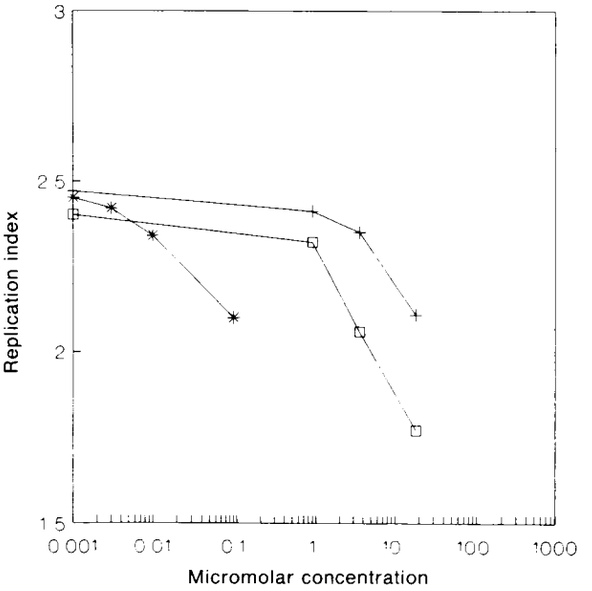

Figure 2. Effects of bleomycin (*), cisplatin (□) and melphalan (+) on the replication index of cultured human lymphocytes. Each point represents the mean value of four independent experiments.

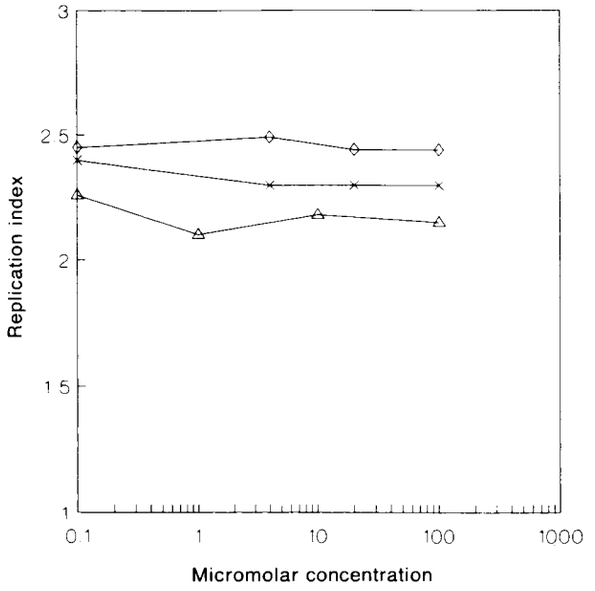

Figure 3. Effects of 6-mercaptopurine (×), 5-fluorouracil (◇) and methotrexate (△) on the replication index of cultured human lymphocytes. For explanation see Figure 2.

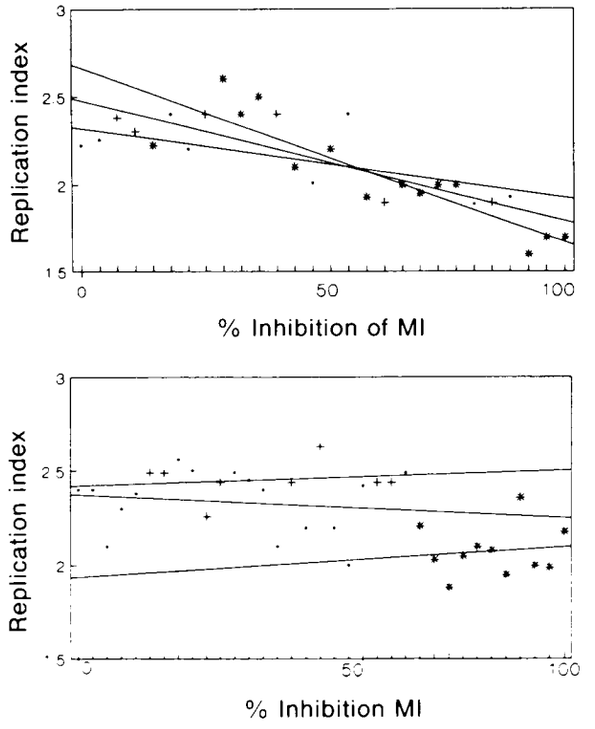

Figure 4. Correlation between replication index and inhibition of MI values from cultures treated with (top panel) melphalan (·), bleomycin (+) and cisplatin (*) and (bottom panel) 6-mercaptopurine (·), 5-fluorouracil (+) and methotrexate (*).

other hand, the CPK allows us to differentiate between drugs which induce cell death and those which have a cytostatic effect by delaying any phase of the cell cycle.

Our results demonstrate an inhibition of the MI by all drugs tested; with all drugs except methotrexate, this inhibition was dose related. Methotrexate causes a decrease of the MI at low concentrations (0.1 μM) and this effect is maintained at higher concentrations. These findings are in agreement with those by Prescott[17] who suggested the use of methotrexate as an agent for synchronizing the proliferation of cells, since it arrests cells in the G_2 phase.

The CPK, on the other hand, was affected in different ways depending upon the mechanism of action of the drug tested. Drugs which directly interact with the DNA produced a CPK delay (Figure 2). By contrast, 5-fluorouracil, 6-mercaptopurine and methotrexate, which do not interact directly with DNA, had no effect on the CPK (Figure 3).

The cytostatic activity exhibited by cisplatin, bleomycin and melphalan could be due to a delay of the cell cycle during DNA damage repair.[18,19] It has been suggested that chemicals which do not cause severe DNA damage, such as methotrexate, 6-mercaptopurine and 5-fluorouracil, probably exhibit a threshold response.[20] In agreement with this, in our study the culture time may not have been sufficiently long to detect an effect on CPK. It must be noted that the highest concentrations used of 5-fluorouracil and methotrexate induced a MI inhibition of 70% without causing any delay on the CPK.

If the correlation between the inhibition of the MI and the CPK is analyzed graphically, the cytostatic activity of DNA-specific agents can be clearly characterized in the system, since they have a negative slope (Figure 4). This behavior could also be used to predict the method of action of unknown compounds.

According to these results a dose-related retardation of the CPK could be interpreted as a potential capacity of drugs to interact directly with DNA; these kind of agents are known to be clinically most effective as antineoplastic agents.[21]

The data presented indicate that the MI and the CPK are sensitive parameters for detection of cytostatic activity. Are both parameters specific for antineoplastics? The answer is no, since various reports indicate that a wide range of chemical, physical and biological factors induce MI inhibition and CPK delay.[11,22,23] Nevertheless, these para-

meters could be used in early steps of the preclinical screening for antitumor activity where a high specificity is not required but reproducibility and sensitivity are desired.[24] In this study we have used PHA-stimulated human lymphocytes as the cellular model. However, other cells which represent more closely the kinetics of tumoral growth should also be used.

References

1. Freshney RJ. *Culture of animals cells: a manual of basic techniques.* New York: AR Liss 1987; 385.
2. White, RJ *In vitro* prescreens. In: Fidler IJ and White RJ, eds. *Design of models for testing cancer therapeutic agents.* New York: Van Nostrand Reinhold 1981: 1–11.
3. Salomon SE, Hamburger AW, Soehnlen B, *et al.* Quantification of differential sensitivity of human tumor stem cell to anticancer drugs. *N Eng J Med* 1978; **298**: 1321–7.
4. Bertelsen CA, Scridak VK, Mann BD, *et al.* Chemosensitivity testing of human solid tumors. A review of 1582 assays with 258 clinical correlations. *Cancer* 1984; **53**: 1240–5.
5. Wiesenthal LM, Marsden JA, Dill PL, *et al.* A novel dye exclusion method for testing '*in vitro*' chemosensitivity of human tumors. *Cancer Res* 1983; **43**: 749–57.
6. Editorial. Clonogenic assays for the chemotherapy sensitivity of human tumors. *Lancet* 1982; **3**: 780–1.
7. Hager T. Cancer drug sensitivity test has problems. Medical News, *J Am Med Ass* 1982; **248**: 3079–83.
8. Ostrosky P, Montero R, Hernßndez L, *et al.* Lymphocyte proliferation kinetics as a cytostatic screening system. *Environ Mol Mutagen* 1988; **11**: 80 (Abstr).
9. Tice R, Schneider L, Rary JM. The utilization of bromodeoxyuridine incorporation into DNA for the analysis of cellular kinetics. *Exp Cell Res* 1976; **102**: 232–6.
10. Bianchi NO, Lezana E. Kinetics of lymphocyte division in blood cultures studied with the BrdU-Giemsa technique. *Experientia* 1976; **32**: 1257–8.
11. Crossen PE, Morgan WF. Analysis of human lymphocyte cell cycle time in culture measured by sister chromatid differential staining. *Exp Cell Res* 1977; **104**: 453–7.
12. Rojas E, Montero R, Herrera LA, *et al.* Are mitotic index and lymphocyte proliferation kinetics reproducible endpoints in genetic toxicology testing? *Mutat Res* 1992; **282**: 283–6.
13. Montero R, Gonsebatt ME, Gerson R, *et al.* AS-101: a modulator of *in vitro* T-cells proliferation. *Anti-Cancer Drugs* 1993; **4**: 351–4.
14. Perry P, Wolff S. New Giemsa method for the differential staining of sister chromatids. *Nature* 1974; **251**: 156–8.
15. Yamane T. *Estadostica.* Mexico: Harla: 1979: 771.
16. Rao AP, Rao P. The cause of G_2-arrest in chinese hamster ovary cells treated with anticancer drugs. *JNCI* 1976; **57**: 1139–43.
17. Prescott DM. *Reproduction of eukaryotic cells.* New York: Academic Press 1976: 117.
18. Mamuris Z, Pricuir M, Dutrillaux B, *et al.* Chromosomal aberrations in lymphocytes of patients treated with melphalan. *Int J Cancer* 1988; **42**: 133–9.
19. Gebhart E, Windolph B, Wopfner F. Chromosomes studies on lymphocytes of patients under cytostatic therapy. II. Studies using the BUDR-labelling technique in cytostatic therapy. *Human Genet* 1980; **56**: 157–67.
20. Scott D, Galloway S, Marshall R, *et al.* ICPEMC genotoxicity under extreme culture conditions. *Mutat Res* 1991; **257**: 147–204.
21. Bloch AW. Antitumor agents and molecular basis of cancer. In: Molecular basis of cancer, part B: macromolecular recognition, chemotherapy and immunology, New York: AR Liss 1985: 207–13.
22. Gonsebatt ME, Mutchinick O. Human lymphocyte proliferation kinetics in Hank's BSS supplemented with autologous plasma and in synthetic medium. *Mutat Res* 1990; **243**: 255–8.
23. Morimoto K, Wolff S. Increase of sister chromatid exchanges and perturbations of cell division kinetics in human lymphocytes by benzene metabolites. *Cancer Res* 1989; **40**: 1189–93.
24. Venditti JM. Preclinical drug development: rationale and methods. *Semin Oncol* 1981; **8**: 349–61.

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