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Suppression of *in vitro* peripheral blood lymphocyte mitogenesis by cytotoxic drugs commonly used in the treatment of breast cancer: a comparative study

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Abstract

Cultures of normal donor peripheral blood mononuclear cells were tested *in vitro* for suppression by chemotherapeutic agents or their metabolites. The drugs tested were those commonly used in the treatment of breast cancer, namely, 5-fluorouracil, doxorubicin, vincristine, methotrexate and cyclophosphamide (actually testing its active metabolite, 4-hydroxy-cyclophosphamide). The lymphocytes were stimulated by phytohaemagglutinin (PHA), and the inhibitory effect of the drugs on subsequent DNA synthesis was measured by tritiated thymidine uptake. Drug concentrations used were equivalent to expected *in vivo* plasma and body fluid levels following i.v. injection of a standard therapeutic dose. Results suggest that the drugs may be ranked for suppression of T-cell function as follows:
doxorubicin > vincristine = cyclophosphamide > 5-fluorouracil > methotrexate.

Introduction

Postoperative adjuvant chemotherapy is being used with ever increasing frequency in the management of malignant diseases, especially breast cancer. Cytotoxic drugs exert their beneficial effect by directly suppressing the proliferation of tumour cells. As yet, such drugs cannot be precisely targeted to malignant cells and so they simultaneously prevent or slow growth in rapidly dividing normal tissues, including those of the immune system. In the chemotherapy of macrometastases, immunosuppression secondary to treatment may be relatively unimportant, since long survival is unusual. However, where bulk tumour has been removed, adjuvant treatment aimed at eradicating residual tumour and micrometastases is frequently used. The cytotoxic drugs most commonly used in adjuvant treatment have been shown to affect the proliferation of normal cells of the immune system [1–6]. It is possible that the disadvantages of drug-induced inhibition of non-malignant T-cell growth and differentiation may outweigh the advantages of cytotoxicity on malignant cells, and indeed, patient prognosis appears to be related to T-cell competence tested both *in vivo* and *in vitro* [2, 6–9]. However, animal studies suggest that some antineoplastic agents which, used at therapeutic doses are cytotoxic to tumour cells, may not cause significant suppression of T-cell function [10]. Theoretically, these would be the drugs of choice for adjuvant treatment. We have therefore attempted a comparative evaluation of the drugs most commonly used in the adjuvant therapy of breast cancer in terms of their *in vitro* effect on normal T-cell proliferation. Designing chemotherapy regimes on the basis of *in vitro* studies of tumour cell cytostatic and cytotoxic effects has proved difficult since human tumours are often characterised both by relatively

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low numbers of proliferating cells and by a heterogeneous population of cells that show marked differences in their response to drugs [11]. However, the anti-proliferative effect of such agents on non-malignant lymphoid cells is more readily investigated.

Peripheral blood lymphocyte cultures have been used extensively as model systems for investigating the regulation of cell activation, growth and proliferation. After isolation, such cells represent resting or G_0 cells. On stimulation with T-cell mitogenic lectins such as phytohaemagglutinin (PHA) resting T-lymphocytes undergo transformation to the G_1 state, initiating the complex sequence of events resulting in proliferation [12]. Since chemotherapeutic agents exert their maximum suppressive effect on cycling rather than resting cells [11], this study has used PHA-activated cultures of non-malignant immunocompetent T-cells in order to investigate the relative inhibitory effect of five different cytotoxic drugs frequently used as adjuvant therapy in breast cancer, on DNA synthesis as measured by tritiated thymidine incorporation.

Materials and methods

Drugs

Five drugs were studied, namely, 5-fluorourcil (Roche Products Ltd. U.K.), doxorubicin (Pharmitalia Carlo Erba), methotrexate (Lederle Laboratories), vincristine sulphate (Eli Lilly and Co. Ltd.), and cyclophosphamide. Since cyclophosphamide is inactive until metabolised in the liver, it was used as its active metabolite, 4-hydroperoxycyclophosphamide (4-HPC), kindly donated by Boehringer Ingelheim Ltd. The first four drugs were tested at concentrations (B) calculated as the maximum expected body fluid levels (μg/ml) following standard i.v. doses [13]. These concentrations are of the same order as tumour inhibitory levels *in vitro* [14–18]; tests were also performed at concentrations of $\times 10^{-1}$ and $\times 10$ of concentration B (concentrations A and C respectively) (see Table 1). The highest concentrations tested (C) were of the same order as peak blood levels achieved in patients after standard therapeutic doses [19–22]. In human subjects, plasma levels of 4-HPC were between approximately 3.5 and 1.4 μg/ml during the first four hours after intravenous injection of 20 mg/kg of cyclophosphamide, [23]; mean peak levels of unbound alkylating metabolites were 12.9 μg/ml/g. of cyclophosphamide administered [24]. Therefore, 4-HPC was tested at concentrations of 0.1 (A), 1.0 (B), and 10 (C) mg/ml.

Preparation of cultures

Lymphocytes from 22 healthy volunteers, aged 18–60 were separated from peripheral blood by density sedimentation (Histopaque 1077, Sigma) and cultured in TC199 culture medium (Wellcome Laboratories) with antibiotics (200 i.u. penicillin and 100 i.u. streptomycin/ml) as previously described [25]. Briefly, quadruplicate cultures

Table 1
Concentrations of cytotoxic drugs added to PHA-stimulated PBL cultures and related to maximum expected body fluid levels and peak plasma levels after administration of a standard i.v. dose *in vivo*.

Drug	Standard i.v. dose (mg.)	Concentration A (μg/ml)	Maximum expected body fluid level (μg/ml)	Concentration B (μg/ml)	Peak plasma levels (μg/ml)	Concentration C (μg/ml)
Doxo	50	0.1	1.0	1.0	3.3	10
5-FU	1000	2.0	20	20	160	200
MTX	50	0.1	1.0	1.0	2.5–6.0	10
VCT	20	0.004	0.04	0.04	0.33	0.4
4-HPC	(1000 mg. cyclophos)	0.1	(see text)	1.0	(13) (metabolites)	10
References			13, 23		19-22, 24	

containing 1×10^6 lymphocytes, 20% autologous plasma and 3 µl PHA (PHA-p, Difco, reconstituted as directed) in a final volume of 3 ml culture medium were set up in tissue culture tubes (Nunc), gassed with 7.5% CO_2, tightly stoppered and incubated at 37 °C. The concentration of PHA used was that which had previously been determined in our laboratory as optimal for this culture system.

Addition of drugs

Drugs were prepared immediately before use by dissolving in the diluent normally used for i.v. injection. Diluent alone was added to control cultures.

Other workers have shown that after 24 hours culture with PHA most T-cells are activated without having proceeded to mitosis [26]. In this study, cytotoxic drugs were added to PHA-activated cultures at 26 hours, since preliminary studies had indicated this as the earliest period of substantial DNA synthesis before the first mitotic division occured.

High plasma levels of free drug are maintained *in vivo*, and therefore in contact with peripheral blood lymphocytes, for a comparatively short time after administration (see Table 1). An *in vitro* contact time of 30 minutes was arbitrarily chosen after initial studies had indicated no significant difference in DNA synthesis following 15, 30 and 60 minutes contact between drugs and lymphocytes. Cells were then washed twice in TC199, resuspended in TC199 plus 20 per cent plasma and the cultures continued for a total period of 72 hours.

Measurement of incorporation of tritiated thymidine (DNA synthesis)

After 68 hours incubation 3 replicates were pulsed with 0.3µCi tritiated thymidine (0.3 µCi, specific activity 1 Ci/mmole), and the cultures incubated for a further 4 hours. One replicate from each group was used to test for viability using the Trypan blue exclusion test. Cultures were harvested and stimulation calculated as the mean molar uptake of radioisotope per unit number of lymphocytes as previously described [27]. Since biological inhibition is an exponential function of drug dose, drug-induced inhibition (DII) was expressed as

$$DII = \log (\text{test result/control})$$

Results

Viability

The viability of lymphocytes cultured with doxorubicin, concentration C was only 14 per cent. For all other concentrations, and for all other drugs tested, no significant loss of viability was observed.

Effect of drugs on mitogenesis

The results are summarised in Table 2 and Figures 1–6. Methotrexate produced no inhibition of DNA synthesis at any concentration tested.

5-fluorouracil produced significant inhibition at concentration C ($p < 0.001$) but not at concentrations A or B. Vincristine and 4 HPC gave comparable results, with modest inhibition at concentration B ($p < 0.05$), and substantial inhibition at concentration C ($p < 0.001$).

Doxorubicin produced substantial inhibition at concentration B ($p < 0.001$) and greater inhibition, largely as a result of cell death, at concentration C ($p < 0.001$).

Table 2
Inhibition of tritiated thymidine uptake (picomoles/10^6 lymphocytes) in PHA transformed lymphocyte cultures by different concentrations of cytotoxic drugs commonly used in the treatment of breast cancer. Results expressed as mean of 22 experiments.

Drug	Control	Drug concentrations		
		A	B	C
Doxorubicin	936	904	593 $p < 0.001$	61 $p < 0.001$
5-Fluorouracil	1082	961	1067	670 $p < 0.001$
Methotrexate	833	785	808	853
Vincristine	1178	1128	990 $p < 0.05$	450 $p < 0.001$
4-HPC	774	684	597 $p < 0.025$	230 $p < 0.001$

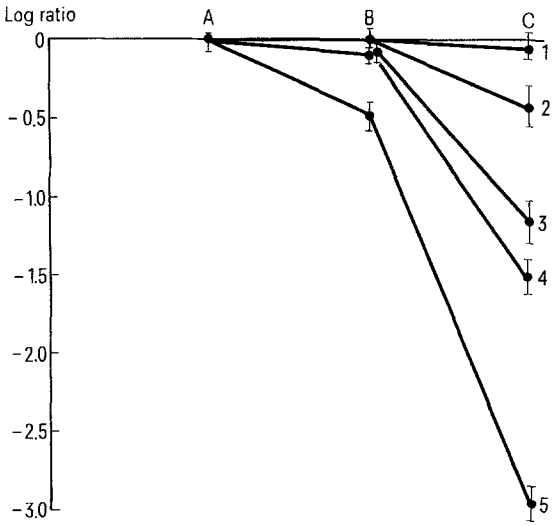

Figure 1
Results of lymphocyte responsiveness in the presence of different concentrations (A, B, and C) of methotrexate (1), 5-fluorouracil (2), vincristine (3), 4-HPC (4) and doxorubicin (5), expressed as means and standard errors of 22 experiments.

Inter-drug comparisons showed that at concentration B there was no significant difference between methotrexate and 5-fluorouracil, nor between vincristine and cyclophosphamide, (though the latter two drugs were more suppressive than the former); doxorubicin produced substantially greater suppression than the other drugs ($p < 0.01$ F test). At concentration C, 5-fluorouracil was more suppressive than methotrexate ($p < 0.01$ F test); cyclophosphamide and vincristine were comparable, and both were significantly more suppressive than 5-fluorouracil, whilst doxorubicin had a substantially greater effect than any of the other drugs ($p < 0.001$ F test).
These results indicate that the relative order of inhibitory effect of the drugs on DNA synthesis in PHA-activated peripheral blood lymphocytes from normal subjects may be ranked as follows:

Doxorubicin > vincristine = 4-HPC
> 5-fluorouracil > methotrexate

Discussion

Although adjuvant chemotherapy for "early" breast cancer is known to prolong the disease-free interval, its efficacy in prolonging overall survival time in these patients has been disputed [28]. Some animal and human studies have actually shown increased metastatic spread of tumours in subjects treated with cytotoxic drugs, and this may be attributed to the potent suppressive effect of these drugs on circulating non-malignant immune cells [29–31]. Many studies have used *in vitro* systems to investigate the sensitivity of tumour cells to different chemotherapeutic agents [14–18]. This study describes a simple and reproducible *in vitro* system to study the relative sensitivity of immunocompetent cells to such agents.
The majority of chemotherapeutic agents exert their maximum cytostatic effect on cycling rather than resting cells [11]. *In vivo* studies have also shown that those drugs most commonly used in adjuvant therapy have their optimum suppressive effect on DNA synthesis when given following an antigenic stimulus and that it is differentiating and proliferating cells which are most sensitive to their action [32]. In addition, some agents have their maximum effect at different phases of the cell cycle, and most of the drugs used in this study act on more than one phase; thus methotrexate targets cells in G_1 and S phase, doxorubicin acts on S phase through to mitosis, 5-fluorouracil is active at all stages of the cell cycle, vincristine acts on the transition from S to G_2 and cyclophosphamide (4-HPC) on the transition from G_1 to S [33]. The culture system described uses activated T-cells cycling from G_1 to mitosis, and drug concentrations likely to be achieved *in vivo* and it is therefore unlikely that the observed results are artefactual.
It is interesting that previous studies of delayed hypersensitivity skin-testing in patients receiving various types of chemotherapy including 5-fluorouracil, vincristine, cyclophosphamide and doxorubicin have shown temporary impairment of primary and established T-cell function [1, 2, 6, 10, 34, 35], although this was not observed in patients receiving only methotrexate and corynebacterium [36]. These *in vivo* results parallel the findings of the present study, where an *in vitro* culture system using normal donor lymphocytes has been used and indicate that the system may be helpful in identifying those chemotherapeutic agents for use in adjuvant therapy which are least likely to cause suppression of non-malignant

T-cell proliferation. Thus our results indicate that doxorubicin is probably best avoided in adjuvant treatment, whilst methotrexate is less likely to produce any significant suppression at the doses commonly used. Further studies of a wider range of drugs and of combinations of drugs are indicated.

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