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On Heterogeneity of Non-Hodgkin's Lymphomas as Regards Sensitivity to Cytostatic Drugs

An *In vitro* Study*

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Abstract—*In the present paper a test model was used to examine whether human non-Hodgkin's lymphomas are heterogeneous with regard to sensitivity to cytosine arabinoside, melphalan, vincristine sulphate and prednimustine* in vitro. *The effects of the four drugs were measured as the differences in incorporation of tritiated thymidine in drug-containing tubes and control tubes. It was found that different parts of three lymphomas out of seven differed significantly in their sensitivity to the same cytostatic treatment* in vitro. *A suggestive correlation was found between the therapeutic effect of prednimustine and its effect in the* in vitro *test model.*

INTRODUCTION

COMBINATION chemotherapy of human non-Hodgkin's lymphomas can induce objective remission in the majority of these patients [1–4]. The response of other solid human tumours to cytostatic treatment is much more variable. Some tumours are initially totally resistant and others show regressions of variable duration. This variation in therapeutic response may have different explanations, e.g., differences in the tumour–host relationship, differences in growth fraction, and differences in the proportion of sensitive and resistant cell clones.

Cytogenetic and cytochemical studies have demonstrated that experimental and human tumours can be composed of more than one cell clone [5–8]. We found that different biopsies from some methylcholanthrene induced sarcomas in mouse [9, 10] and some human adenocarcinomas of the colon and the stomach differ markedly in their sensitivity to the same cytostatic treatment *in vitro* [11]. Studies on human non-Hodgkin's lymphomas are, however, consistent with the monoclonal origin of these tumours [12], and it is therefore of interest to study whether they show lower variability concerning sensitivity to cytostatic drugs than the solid tumours.

This was done in the present work using an *in vitro* model.

MATERIALS AND METHODS

Lymphomas from seven patients (Table 1) were used in this study. Each lymph node was divided into four pieces, each about $10 \times 10 \times 10$ mm in size, one for the histopathological diagnosis and the others were tested separately for their sensitivity to cytostatic drugs. From the latter three pieces, small biopsies about $2 \times 2 \times 2$ mm in size were also taken for histopathological diagnosis. Details of the preparation and incubation procedures have been described before [13, 14]. The tumour material was cut with scissors in Parker 199 (SBL, Stockholm) and brought into a suspension of single cells and small tissue fragments according to the method described by Borell [15]. The cells were washed once in Parker 199, then resuspended in incubation medium: Parker 199, containing 20% human serum, heparin 50 U/ml, sodium benzyl penicillin 50 i.u./ml, and streptomycin sulphate 10 μg/ml.

The cytostatic drugs were added to give the following final concentrations: cytosine arabinoside 0.025 mg/ml (Sigma, St. Louis), melphalan 0.2 mg/ml (Alkeran, Burroughs &

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Table 1. *Presentation of the histologic type of the lymphomas and the effect of prednimustine treatment* in vivo *and* in vitro

Patient No.	Histologic type (Rappaport)	Stage (Ann Arbor)	Prednimustine treatment		
			Therapy dose	Effect *in vivo*	Effect *in vitro**
1	Undifferentiated lymphoblastic	II	60 mg daily	Progressive disease	16.53
2	Diffuse histiocytic	III	120 mg daily for 5 days	Progressive disease	29.45
3	Diffuse histiocytic	III	60 mg daily	Complete remission of short duration	41.18
4	Diffuse lymphocytic, poorly differentiated	IV	60 mg daily	Complete remission more than 2 yr	62.34
5	Diffuse mixed	III	—	—	95.52
6	Nodular lymphocytic, poorly differentiated	III	200 mg daily for 5 days every other week	Complete remission more than 2 yr	102.36
7	Nodular lymphocytic, poorly differentiated	III	60 mg daily	Complete remission more than 2 yr	75.79

*Mean of the effect values of the three biopsies from each lymph node.

Wellcome, London), vincristine sulphate 0.1 mg/ml (Oncovin, Lilly, Indianapolis), and prednimustine* 0.1 mg/ml (Leo, Helsingborg). The concentrations of the cytostatic drugs used in the *in vitro* test in the present investigation were chosen from the results obtained on normal mouse thymocytes [13] and on methylcholanthrene induced sarcoma cells [16]. Undoubtedly the concentrations used are high compared to these reached *in vivo* during therapy. However, we have showed that there is a correlation between our *in vitro* model and the response *in vivo* in serially transplanted methylcholanthrene induced mouse sarcomas [16, 17]. The *in vitro* method thus estimates a tumour cell characteristic that has a biological meaning also at *in vivo* conditions.

Solutions of melphalan and prednimustine were freshly prepared for each experiment. The other drugs were diluted from stock solutions kept frozen. All incubations were carried out in the medium described above, and were set up within 90 min after operation. The tumour cells were incubated with cytostatic drugs for 3 hr. Then ^{3}H-thymidine (^{3}H-TdR, methyl-^{3}H-thymidine, spec.act. 1.9 Ci/mmole, Schwarz/Mann, Bio Research Inc., Orangeburg) was added to give a final concentration of 2 μCi/ml and the incubation was continued for another hour. Controls were set up in each series of experiments. These received only ^{3}H-TdR.

The major mechanism of action of cytosine arabinoside is a direct interference with DNA synthesis through an inhibition of the conversion of cytidine to deoxycytidine, melphalan and prednimustine acts as an alkylating agent. The main point of attach is suggested to be on the nucleic acids. ^{3}H-TdR is therefore the best parameter for these drug effects. The mechanism by which vincristine exerts its cytostatic effect is chiefly by a blockage of mitosis as a result of the inactivation of the mitotic spindle. The use of vincristine, in *in vitro* tests such as ours has been criticized as this major effect of vincristine in therapeutic concentrations cannot interfere with DNA synthesis during a few hours incubation. The mode of action in the *in vitro* tests must be different. Richards *et al.* [18] used quite similar concentrations to ours and could demonstrate a depression of ^{14}C formate incorporation into nucleic acids both *in vitro* and *in vivo*. We have also used different labelling precursors to test the effect of vincristine and it abolished the incorporation of 3H-TdR and 5-^{3}H-UR at the same concentration [13] but at the lower concentrations tested the incorporation of ^{3}H-TdR was more inhibited than the incorporation of 5-^{3}H-UR [13]. We have therefore, for all four tested drugs, used ^{3}H-TdR incorporation as the only parameter for drug effect. All tests were performed in duplicate. Preparation of cells and determi-

*Prednimustine (a chlorambucil ester of prednisolone) was kindly supplied by AB Leo, Helsingborg, Sweden.

nation of DNA and radioactivity has previously been described in detail [13, 14]. DNA synthesis was expressed as precursor incorporation per DNA units. In order to obtain near-normally distributed variates suitable for statistical analysis, the following expression was used

$$a = 100 \times \log_{10} \frac{\text{counts/min} \times 10^4}{(\text{AES}) \times (\text{DNA})}$$

where (counts/min) is the number of counts registered, (AES) is the counts/min registered with automatic external standardization and (DNA) is the amount of DNA in the sample expressed in OD_{490} units corrected for the standard curve. The effect of cytostatic drugs is expressed as the difference between the mean *a*-value in the two control tubes and the mean *a*-value in the two tubes where cells were exposed to cytostatic drugs.

Statistical considerations

The total variance in the material consists of technical "error" variance (V_E), of variance between "drug effects" (V_D), of variance between "biopsies" (V_B), and of interaction between the latter two variances (V_I).

V_E can be estimated from 104 double determinations and is found to be 87.75—which means that the error of the difference between two double determinations (e.g., control and cytostatic drug) is $\sqrt{87.75\,(\frac{1}{2}+\frac{1}{2})}=9.37$ and an "effect" value of 24.58 is thus significant at the 0.1% level. Only lymphoma 2 had a significantly larger error variance (as studied in a Bartlett's test) which was consequently used in statistical calculations including this lymphoma.

The degree of heterogeneity within a tumour can be studied by comparing V_I with V_E—if the former is significantly larger than the latter, the various biopsies from this tumour vary significantly, the tumour is heterogeneous.

V_I can also be studied for the total material of biopsies for each drug–irrespective of the "tumour" to which the biopsies belong. This interaction is made up of two parts: that just discussed and which can be characterized as "within tumour" interaction, and a part consisting of the "between tumour" interaction and which expresses the variability in heterogeneity between tumours. The statistical significance can be tested against the "within tumour" interaction.

Each tumour was tested simultaneously with the four cytostatic drugs, utilizing the same control group. In order to explore the possible correlation in response to the four drugs, their effects were compared pair-wise using a test for partial correlation.

The three variates determined for each tumour—namely, the *a*-values in control tubes, in tubes containing cytostatic drug 1, and in tubes containing cytostatic drug 2—will be called x_k, x_1, and x_2, respectively. To study whether the effects of the two drugs co-vary irrespective of the variation in control tubes, the partial correlation coefficient $r_{1;2;k}$ was determined as:

$$r_{1;2;k} = \frac{r_{1;2} - r_{1;k} \times r_{2;k}}{\sqrt{(1-r_{1;k}^2)(1-r_{2;k}^2)}}$$

where the indices to the correlation coefficients mark the variates upon which they are determined.

RESULTS

The incorporation of ^{3}H-TdR was markedly reduced when the lymphomas were treated with cytosine arabinoside (Fig. 1a) or melphalan (Fig. 1b). A marked reduction was also found in the majority of the lymphomas when treated with vincristine (Fig. 1c) or prednimustine (Fig. 1d). The cytostatic effect is expressed as the difference between the *a*-values in the controls and the drug treated cells. The statistical significance of these effects was calculated as described above (see "statistical considerations").

There seems to be differences in sensitivity to cytostatic drugs between biopsies within tumours, that is, the tumours appear to be heterogeneic. The statistical significance of the heterogeneity of a certain tumour when tested with a certain drug is shown in Fig. 1 as variance ratios (F) between the V_I and V_E variances (see "statistical considerations").

The sensitivity to cytostatic drugs *in vitro* differs significantly between different pieces from some of the tumours: No. 1 when tested with vincristine, No. 3 when tested with vincristine and cytosine arabinoside, and No. 7 when tested with melphalan and prednimustine. No significant heterogeneity could be demonstrated in the other four lymphomas when tested with any of the four drugs, but it cannot be proved that the various lymphomas actually differ in degree of heterogeneity as only three pieces of each tumour was tested. Table 2 shows that the average heterogeneity of the lymphomas reaches statistical signific-

Table 2. Variance analysis of contribution to the interaction (between the two sources of variation "biopsies" and "drug effects") of two sources: tumour (="between tumours") and biopsies from the same tumours (="within tumours")†

	Cytosine arabinoside			Melphalan			Vincristine sulphate			Prednimustine		
Source of variation	d.f.	Variance	F	d.f.	Variance	F	d.f.	Variance	F	d.f.	Variance	F
Within tumours	14	191.73*		14	169.04*		14	285.96***		14	177.26*	
Between tumours	6	3289.47	17.15***	6	4050.84	23.96***	6	1471.17	5.14**	6	3242.40	18.29***

†The significance of the latter is tested against general error variance.

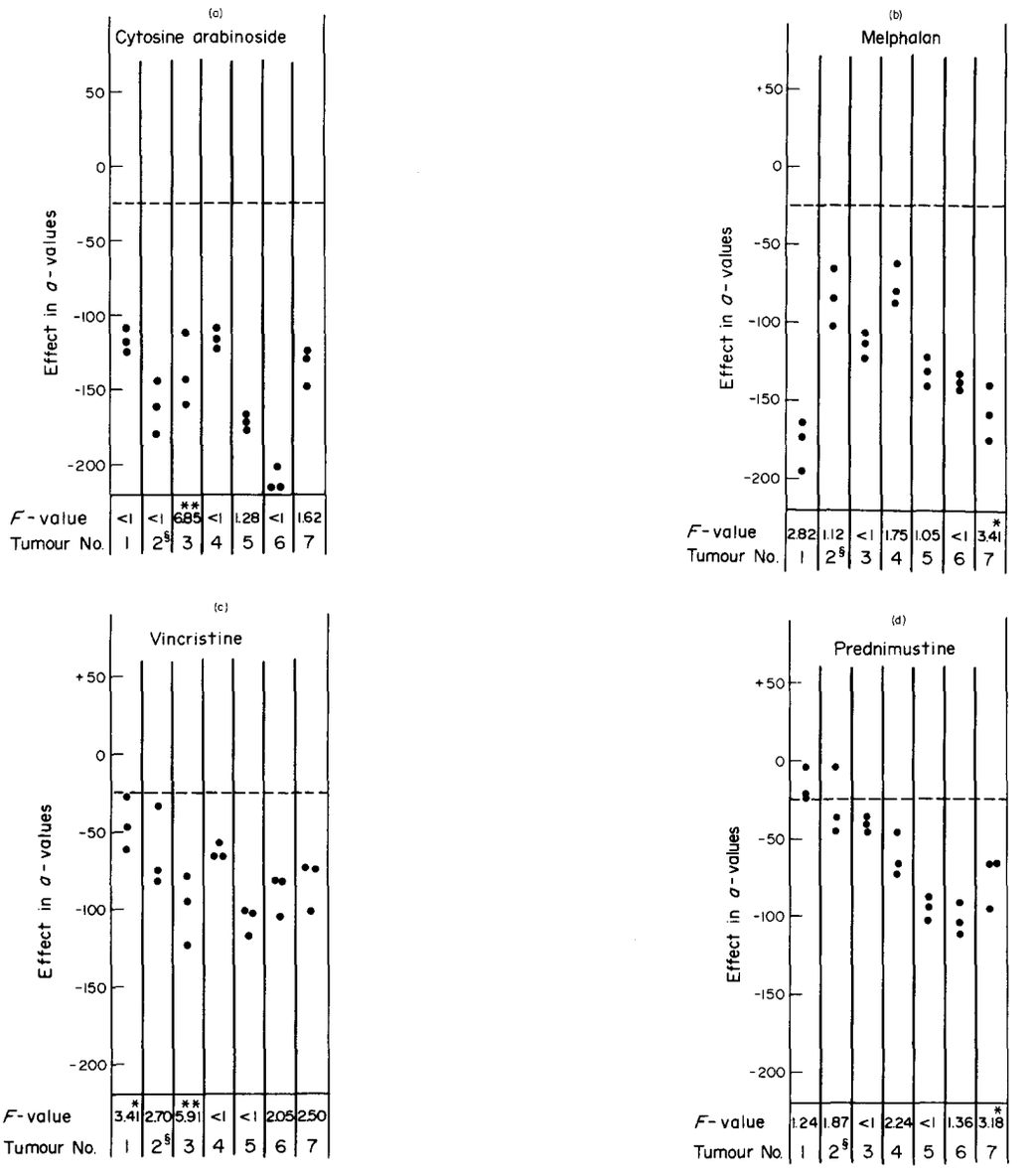

Fig. 1. *Differences in sensitivity to cytostatic treatment* in vitro *between different biopsies from lymphomas. Effect expressed as difference in* a*-values between controls and drug-treated cells. Three biopsies (each represented by a cross) from each tumour. The* 0.1% *significance level is marked as a dashed line in the figure,* § *marks tumours where the error variance of these tumours had to be used instead of the general error variance. For further explanation, see the text.*

ance for all four cytostatic drugs. Table 2 also demonstrates that the different lymphomas showed a signficantly greater variability in response than did the biopsies studied from each tumour.

Table 3 shows the partial correlation coefficients determined pairwise between the various drugs. Cytosine arabinoside and prednimustine show a statistically significant correlation, indicating cross-resistance between these two drugs.

DISCUSSION

Different pieces from the same lymphoma of some patients showed differences in their sensitivity to cytostatic drugs *in vitro*. Thus, heterogeneity was demonstrated in two lymphomas out of seven when tested with vincristine, and in one lymphoma out of seven when tested with cytosine arabinoside, melphalan or prednimustine. In a previous paper [11] the heterogeneity of human adenocarcinomas of the colon and the stomach were studied in a similar *in vitro* test model. Four colon cancers out of eleven were found to be heterogeneous when treated with melphalan but only one showed heterogeneity when treated with cytosine arabinoside. Three out of five cancers of the stomach showed heterogeneity to treatment with cytosine arabinoside, whereas none

Table 3. Correlation between the effects of the four cytostatic drugs

Cytostatic drugs compared	Partial correlation coefficient	*t*-value	*P*
Melphalan—cytostine arabinoside	0.33	0.71	NS
Melphalan—vincristine	−0.48	−1.10	NS
Melphalan—prednimustine	0.20	0.40	NS
Cytosine arabinoside—vincristine	0.33	0.71	NS
Cytosine arabinoside—prednimustine	0.91	4.50	$P<0.02$
Vincristine—prednimustine	0.61	1.55	NS

Calculated as partial correlation coefficients between mean ^{3}H-TdR incorporation in tubes containing the cytostatic drugs, eliminating incorporation in control tubes.
t-test of coefficients $\neq 0$.

of these tumours were found to be heterogeneous to melphalan treatment. The pattern of heterogeneity as regards sensitivity to cytostatic drugs might be different in different types of solid tumours. However, the material has to be extended to study this possibility.

Recently, a difference in drug sensitivity has also been found in three cell clones derived from a heteroploid tumour cell line [19]. However, these cell clones also differed morphologically and consisted of fibroblast-like cells, epithelial-like and round cells and giant cells. In the present material different biopsies from the same lymphomas showed identical histological pictures.

It appears that the *in vitro* test employed can demonstrate differences between different cell clones which are not observable in ordinary histopathological appearance. They may represent differences in drug penetration of cell membrane, intracellular drug inactivation, in repair enzyme system development. Other explanations to differences in sensitivity to the drugs between different biopsies might be due to local nutritional differences resulting in variations in the rate of proliferation of the tumour cells. These factors should cause differences in the incubations of ^{3}H-TdR in the control incubations from different biopsies within a tumour. However, no such differences were found in this study.

As pointed out above, all lymphomas were highly sensitive when treated with melphalan or cytosine arabinoside *in vitro*. In contrast to this, several of the adenocarcinomas of the colon and the stomach were found to be resistant to treatment with these drugs *in vitro*. This difference in sensitivity and possible differences in heterogeneity might explain why *in vivo* the cytostatic treatment of lymphomas is much more successful than this treatment of the adenocarcinomas.

Two of the drugs—melphalan and prednimustine—studied in this investigation are derivates of nitrogen mustard and it is well-known that such drugs often show cross-resistance. However, in the test model used here, no correlation could be found between the effects of these drugs. A statistically significant correlation was found between the effects of cytosine arabinoside and prednimustine. The mechanism behind this correlation is for the moment unknown.

Prednimustine is a new efficient cytostatic agent in the treatment of non-Hodgkin's lymphomas [20, 21]. In the present investigation a correlation was suggested between the therapeutic effect of this drug and its effect on the ^{3}H-TdR incorporation of the lymphoma cells *in vitro*.

Six of the patients (all except patient No. 5) received chemotherapy with prednimustine as single agent (Table 1). Three patients (Nos. 1, 2 and 3) were given radiotherapy initially, since their disease at presentation was in stage I or II. After relapse, chemotherapy was started. Only one patient (No. 4) was previously treated with cytostatic drugs (cytoxan, oncovine, and prednisolone), which after two cycles were replaced by prednimustine because of side effects. In two patients (Nos. 1 and 2) prednimustine had no

effect at all on the progression of the disease. It is remarkable that these two lymphomas were the least sensitive ones when tested with prednimustine *in vitro*. A third patient (No. 3) obtained a remission of short duration (2 months) and his lymphoma was only moderately sensitive to prednimustine *in vitro*. The other three patients (Nos. 4, 6 and 7) obtained complete remissions after 2–6 months of treatment and are still in complete remission after more than 2 yr. These lymphomas were also the most sensitive ones when tested with prednimustine *in vitro*.

Two of the patients were treated with prednimustine according to a high-dose intermittent schedule, whereas the other patients were given a daily dose of the drug. The patients are, however, comparable as no difference was found in the therapeutic effect of these drug regimens in a randomized trial [21]. The most responsive lymphomas of this series (Nos. 4, 6 and 7) had "favorable histologies" whereas the unresponsive ones were of the undifferentiated lymphoblastic (Nos. 1 and 2) or diffuse histiocytic (No. 3) types.

One of the lymphomas, where complete remission of long duration was obtained, showed heterogeneity to prednimustine treatment in the *in vitro* test. However, in this lymphoma the incorporation of ^{3}H-TdR was markedly inhibited also in the least sensitive biopsies. Thus, heterogeneity seems to be of importance for the therapeutic effect only when the least sensitive clone is resistant or only moderately sensitive.

As there is often a great degree of heterogeneity in drug sensitivity within mouse and human cells in culture [22], methylcholanthrene induced mouse sarcoma, [9, 10], mouse mammary adenocarcinomas, [23], human adenocarcinomas of colon [11], and now non-Hodgkin's lymphoma, the predictive value of a test performed on a biopsy from such a tumour would be very slight, even if there was a test method which *per se* gave a good correlation to the *in vivo* reaction of the cells to the cytostatic drug under study. Therefore, it seems that the prospect for predictive tests usable in clinical practise is not particularly bright as all *in vitro* methods are based on estimates of the sensitivity of samples removed from a large and often diffusely distributed tumour mass.

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