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Assessment of Drug Activity and Proliferation Ex Vivo for Prediction of Outcome in Aggressive Non-Hodgkin's Lymphomas

Suzanne M. Rehn Ericsson, Rolf L. Larsson, H. Peter Nygren, Christer Sundström and Bengt L.G. Glimelius

From the Department of Oncology, Radiology and Clinical Immunology, Section of Oncology (S.M. Rehn Ericsson, H.P. Nygren, B.L.G. Glimelius), Department of Medical Sciences, Section of Clinical Pharmacology (R.L. Larsson) and the Department of Genetics and Pathology, Section of Pathology (C. Sundström), University of Uppsala, Akademiska sjukhuset, Uppsala, Sweden

Correspondence to: Suzanne Rehn Ericsson, Department of Oncology, Akademiska sjukhuset, SE-751 85 Uppsala, Sweden. Tel: +46 18 6113 186. Fax: +46 18 6115 528. E-mail: suzanne.rehn.ericsson@onkologi.uu.s e

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The activity of cytotoxic drugs and tumour cell proliferation rate were assessed ex vivo using the fluorometric microculture cytotoxicity assay (FMCA) and stainings for Ki67 and mitosis in 40 patients with aggressive non-Hodgkin's lymphomas (NHL). The findings were correlated to clinical response and survival. Twenty-three patients had a complete remission and 10 a partial remission. A drug sensitivity index based on the cell survival for three major drugs in NHL treatment was derived empirically and proliferation was expressed as low-, intermediate- or high. In 5 out of 8 drugs tested, cell survival ex vivo was higher in clinical non-responders than that in responders. Using the median drug sensitivity index as a cut-off, the sensitivity and specificity for tumour response were 58% and 100%, respectively, and was similar for the proliferation index. Both indices combined increased the sensitivity to 73% at retained specificity. Intermediate/high proliferation was significantly associated with impaired survival, whereas the drug sensitivity index was not predictive of survival. Thus, ex vivo assessments of drug sensitivity and proliferation seem to provide prognostic information in aggressive NHL.

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Aggressive non-Hodgkin's lymphomas (NHL) are generally sensitive to both radiotherapy and chemotherapy. In localized disease, radiotherapy gives a high rate of complete responses (CR) with very few relapses within the irradiated site (1–3). Most patients with generalized disease treated with chemotherapy respond to the treatment but a CR is essential to achieve cure. About one-third of patients in CR will have a tumour recurrence (3, 4) but some of these patients could be rescued with renewed treatment, seemingly requiring high-dose therapy with stem cell support (5, 6).

The International Index provides clinical parameters for risk calculation (4). In addition, a number of tumour-related characteristics have been explored for prognostic information. Among these, factors reflecting tumour cell proliferation have often provided prognostic information (7–10).

Another prognostic factor in chemotherapy-treated NHL is tumour inhomogeneity as assessed by magnetic resonance imaging (MRI; 11). However, other factors should also be predictive of response to chemotherapy. An obvious one is intrinsic cell resistance to the drug (12). Several ex vivo methods have been developed to reflect this resistance, one of which is the fluorometric microculture cytotoxicity assay (FMCA; 13). The aim of this explorative study was to assess, ex vivo, the cytotoxic drug sensitivity and rate of proliferation in aggressive NHL and compare the results with clinical outcome with respect to response and survival.

MATERIAL AND METHODS

Patients

Forty patients with aggressive NHL treated with chemotherapy with curative intent were included in the study. Thirty-one tumour samples were taken at diagnosis and 9 at the time of a tumour relapse or tumour progression (denoted 'recurrence'). All patients were classified according to the REAL classification (14). Clinical staging was performed according to the Ann Arbor classification (15). Patient characteristics are presented in Table 1. The follow-up period, from the time the cells were sampled for

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Table 1

Patients' characteristics and distribution according to drug sensitivity and proliferation indices

	Drug Sensitivity Index			Proliferation Index		
	All	<1.5	≥1.5	Low	Intermediate	High
Histology						
DLBCL	26	14	12	9	8	9
PTCL	3	2	1	1	0	2
B-LBL	4	1	3	1	2	1
T-LBL	1	0	1	0	1	0
ALCL	2	0	2	0	1	1
MCL	1	1	0	1	0	0
FCL (Grade III)	2	1	1	0	0	2
Uncl.	1	0	1	1	0	0
Disease status						
Primary	31	16	15	12	8	11
Recurrence	9	3	6	1	4	4
Stage						
I	3	2	1	2	1	0
II	11	5	6	4	4	3
III	11	6	5	4	3	4
IV	15	6	9	3	4	8
Proliferation Index						
Low	13	8	5			
Intermediate	12	6	6			
High	15	5	10			

For definitions of cut-off values for drug sensitivity and proliferation indices, see Materials and Methods.
Abbreviations: DLBCL = diffuse large cell B-cell lymphoma; PTCL = peripheral T-cell lymphoma; LBL = lymphoblastic lymphoma; ALCL = anaplastic large cell lymphoma; MCL = mantle-cell lymphoma; FCL = follicular centre cell lymphoma; Uncl. = unclassifiable.

FMCA examination, ranges from 25 to 178 months (median 62 months).

Treatment

Drugs included in the different chemotherapy combinations are listed in Table 2. Three stage I patients were treated with chemotherapy; two with CHOP and one according to the BFM-83 protocol.

Among patients in stages II–IV, 16 received CHOP and 4 received CHOP with methotrexate in a randomized trial (16); 4 elderly patients received CNOP and 2 patients were given an oral alternative to CHOP. One patient received CHVMP + VB, one was treated with VACOP-B and one with VAD. Two children with lymphoblastic lymphomas received treatment according to the BFM-90 protocol and one child with large cell anaplastic lymphoma was treated according to the BFM-95 protocol. One patient in relapse received MIME. Four patients with relapse were treated with chemotherapy agents in accordance with the results of the FMCA tests, all with cisplatin-containing combinations.

Sampling for the FMCA was always performed prior to the chemotherapy, to which it was subsequently correlated. Clinical response to this treatment was defined either as CR or partial response (PR). Non-responders had stable or progressive disease (7 cases).

Handling of the biopsy material, immunophenotyping

Tumour sampling was mostly part of the routine clinical work-up but was done exclusively for the ex vivo analysis in 11 cases. The sampling was approved by the local ethics committee of Uppsala University Hospital. Part of the material was fixed in neutral buffered formalin for routine histopathology (haematoxylin-eosin, Giemsa and Laidlaw stains). This part was also used for silver staining of mitotic figures and immunostaining with the MIB-1 antibody (see below).

Another part of the material was snap-frozen in isopentan and dry ice and stored at −70°C, for further use. The remaining material was used for the preparation of tumour cells. Part of the cell suspension was used for immunological phenotyping and part was frozen in liquid nitrogen for later ex vivo testing in the FMCA (29 cases) when this was not done immediately (11 cases). Cryopreservation does not seem to affect drug sensitivity of the tumour cells (17).

Table 2

Patient treatment overview according to various drug regimens and their individual drug contents

	n	Cyclo.	Doxo.	Vincr.	Predn	Mitox.	Meth.	Etop	Cytar.	Ida.	Iphosp.	Bleo	Cispl.	M-GAG	Aspara.
CHOP	18	x	x	x	x										
CHOP-M	4	x	x	x	x		x								
CNOP	4	x		x	x	x									
CHOP p.o.	2	x			x			x		x					
CHVMP-VB	1	x	x	x	x		x	x				x			
VACOP-B	1	x	x	x	x			x				x			
VAD	1		x	x	x										
MIME	1						x	x			x			x	
BFM-83	1	x	x		x		x	x	x						
BFM-90	2	x		x	x				x						x
BFM-95	1	x	x		x		x	x	x		x				
FMCA	1							x			x		x		
FMCA	1				x			x					x		
FMCA	1				x				x				x		
FMCA	1								x		x		x		

Abbreviations: n = number of patients; Cyclo. = cyclophosphamide; Doxo. = doxorubicin; Vincr. = vincristine; Predn. = prednisolone; Mitox. = mitoxantrone; Meth. = methotrexate; Etop. = etoposide; Cytar. = cytarabine; Ida. = idarubicin; Iphosp. = iphosphamide; Bleo. = bleomycin; Cispl. = cisplatin; M-GAG = methyl-GAG; Aspara. = asparaginase. p.o. = per oral; FMCA = chemotherapy in accordance with the result of the FMCA test.

Mitotic count

Paraffin-embedded sections were deparaffinized and stained with a modification of Gomori's methenamine-silver method (18) as described by Busch and Vascò (19) to make the mitotic nuclei more prominent. Counterstaining with Ehrlich's haematoxylin was required to identify the non-mitotic nuclei. The number of mitoses was counted using a high-power field lens ($\times 40$) in at least 10 different fields and then divided by the number of counted fields. The cut-off level for low and high mitotic counts was chosen as the median value (2.0 mitoses/high-power field).

Ki-67

Prior to staining with the monoclonal antibody MIB-1 (Immunotech, France), recognizing the Ki-67 antigen present in all cells within the cell cycle, the sections were deparaffinized (20). Immunostaining with monoclonal antibodies recognizing CD45RO (T-cells; UCHL-1, Dako A/S, Denmark) and CD20 (B-cells; L-26, Dako A/S, Denmark) was done to avoid counting non-tumour areas. Substitution of primary antibodies by tris buffer saline was employed for negative controls. Reactive lymph nodes were used as positive controls. The percentage of MIB-1 stained tumour cells was counted in 10 sections of a high-power field in which 100 cells were counted per section. The cut-off level for low and high values was the median value (50%).

Proliferation index

A proliferation index was based on the results of the mitotic count and the Ki-67 value. If both values were below or above their medians, the tumour was regarded to have a low or high proliferation index, respectively. The proliferation index was denoted as intermediate if the mitoses and Ki67 classifications differed. The proliferation index according to patient characteristics is presented in Table 1.

FMCA procedure

The principal steps of the FMCA procedure have been described previously (13, 21). Day one, 180 µl of the tumour cell preparation (5×10^5 cells/ml culture medium) was seeded into the wells of V-shaped 96-well microtitre plates (Nunc, Roskilde, Denmark). The plates were prepared in advance, with drugs tested (Table 3) and kept frozen at -70°C prior to use as previously described (21). The cytotoxic drugs were obtained from commercial sources and were tested at the empirically derived cut-off concentration as described (21). The active metabolite of cyclophosphamide, 4-hydroperoxycyclohosphamide, was used. Each drug was tested in triplicate. Six blank wells received only culture medium and six wells with cells but without drugs served as controls. The culture plates were then incubated at 37°C in humidified atmosphere containing 95% air and 5% CO_2. At the end of the incubation period (72 h), the plates were centrifuged (200 g, 7 min) and the medium removed. After one wash in phosphate buffered saline (PBS), 200 µl PBS containing fluorescein diacetate (FDA) (10 µg/ml) was added columnwise to control, experimental and blank wells. Subsequently, the plates were incubated for 30–40 min before reading the fluorescence in a microplate fluorimeter, Fluoroscan 2 (Labsystems, Oy, Helsinki, Finland). The fluorometer was blanked against wells containing PBS including the dye but without cells. Quality criteria for a successful assay

Table 3

Drugs tested ex vivo and mean cell survival index (SI) values according to clinical response

		Mean SI value (SD)		
	n	Responders	Non-responders	p-value for difference
Cyclophosphamide	38	20% (22)	40% (21)	0.06
Doxorubicin	38	37% (23)	60% (37)	0.04
Prednisolone	38	58% (22)	73% (29)	0.13
Etoposide	32	56% (23)	73% (33)	0.15
Mitoxantrone	37	28% (22)	43% (32)	0.16
Vincristine	39	53% (22)	51% (24)	0.85
Cisplatin	35	55% (24)	42% (10)	0.19
Cytarabine low conc.	29	51% (20)	44% (18)	0.46
Cytarabine high conc.	18	30% (13)	45% (17)	0.06

Abbreviations: SD = standard deviation, n = number, conc. = concentration.

included a fluorescence signal in control cultures of more than $5 \times$ mean blank values and a mean coefficient of variation (CV) in control cultures of less than 30%. Since the FMCA cannot dissociate effects on normal versus tumour cells, a proportion of tumour cells of $\geq 70\%$, as assessed by May-Grünvald Giemsa stained cytocentrifuge samples from control wells, after the 72 h incubation was required for a successful assay. This empirically derived cut-off seems to provide tumour-specific drug sensitivity data (21). The technical success rate using these criteria was 80%.

Quantification of FMCA results

The results obtained by the indicator FDA are presented as the survival index (SI) defined as fluorescence of experimental as a percentage of control cultures with blank values subtracted. The SI reflects the number of living cells (13). Individual drugs were evaluated according to their capacity to distinguish non-responders from responders (CR + PR) in terms of SI (Table 3). Furthermore, the SI values of the drugs considered most relevant in aggressive NHL, i.e. cyclophosphamide, doxorubicin, and prednisolone, which also had an acceptable number of cases, were then used to form a drug sensitivity index. First, the median values and the standard deviations (SD) were calculated for the SI values of the index drugs.

An SI value below the median value for that drug and sample was given a score of 1, an SI value equal to or above the median value but below the median plus one SD was given the score of 2 and an SI value equal to or above the median plus one SD was given the score of 3.

For each sample, the mean value of the individual scores was calculated to form the drug sensitivity index that could thus range from 1.0 to 3.0. The drug sensitivity index according to patient characteristics is presented in Table 1.

Owing to the shortage of cells, FMC results were not always available for all three of the index drugs. Thus, in six cases the index was based on the SI values for two drugs. For correlation of response to treatment and survival, a drug sensitivity index of less than the median value (1.5) was selected as the cut-off value to indicate chemotherapy sensitivity. A drug sensitivity index calculated in the same way but using the drugs the patients actually received was also tested but was found to be less predictive (not shown).

Statistical methods

Differences in the distribution of values between two groups were tested using the two-tailed unpaired t-test, while differences in the distribution of values between different groups were tested with χ^2-test.

Cox's proportional hazards model was used to analyse the effect of different variables on survival with results reported as relative hazards. Patients dying of intercurrent diseases were not included in the population at risk after their death, provided they were in CR.

The sensitivity of an ex vivo analysis was calculated as the percentage of clinical responders (CR or PR) that had an index below the cut-off, and specificity was calculated as the percentage of clinical non-responders that had an index above the cut-off.

RESULTS

Drug sensitivity and proliferation ex vivo and their relation to clinical parameters

There were no differences in drug sensitivity ($p = 0.80$), or proliferation index (low against intermediate/high) ($p = 0.42$) according to stage of the disease (Table 1).

Among the 9 patients who were examined at the time of tumour recurrence all but one had an intermediate/high proliferation compared with 19/31 among those with primary NHL. Six of these patients with recurrences had a high drug sensitivity index compared with 15/31 among the primary NHL patients (Table 1). None of these differences was statistically significant ($p = 0.12$ and $p = 0.33$, respectively).

Only 35% (14/40) of the samples had maximal variation in ex vivo drug activity, i.e. drugs scoring both 1 and 3 (not shown), indicating limiting resistance variation within a sample.

Drug sensitivity ex vivo vs. tumour response

Twenty-three patients (58%) had a CR and 10 patients (25%) a PR, giving a total response rate of 83% (33/40). For cyclophosphamide, doxorubicin and cytarabine at high concentrations, the SI values were higher for non-responders compared with responders (borderline significance; Table 3). Prednisolone etoposide and mitoxantrone also showed a trend toward lower mean SI values for responding versus non-responding patients. SI values for vincristine did not differ between the response groups, whereas for cisplatin and cytarabine at low concentrations, a trend toward paradoxical inverse relationships with higher SI values for responders than for non-responders was observed.

The seven non-responders all had a high drug sensitivity index (≥ 1.5; Table 4). Thus, using this as a cut-off, the specificity was 100%, whereas 19/33 responders had a drug sensitivity index < 1.5 with a sensitivity of 58%. The drug sensitivity index did not dissociate CR from PR ($p = 1.0$; not shown) but statistically significantly dissociated clinical responders from non-responders ($p = 0.006$; Table 4). Five cases had a drug sensitivity index of 3.0. Three patients were non-responders and all three had rapidly progressive disease (PD) with a mean survival time of 2 months. The remaining four non-responders had drug sensitivity indices between 1.5 and 2.7. Three had a short period of stable disease (SD) and one had PD. The mean survival time in this group was 5 months.

Among the 9 patients with tumour recurrence, 3/3 patients with a drug sensitivity index < 1.5 responded to treatment compared with only 2/6 patients with a drug sensitivity index ≥ 1.5 ($p = 0.06$). Two patients with tumour recurrences are still in CR, each with drug sensitivity indices of 1.0.

Proliferation index versus tumour response

Patients with a low proliferation index did not have significantly lower drug sensitivity index values than those with an intermediate/high proliferation index (mean 1.6 vs. 1.8, $p = 0.28$; not shown). Patients with low tumour cell proliferation had significantly higher response rates (13/13) than those with intermediate/high proliferation (20/27; $p = 0.04$; Table 4). Patients with low proliferation had a significantly higher CR rate compared with patients with intermediate/high proliferation (11/13 vs. 12/27, $p = 0.02$; not shown). The proportion of responders identified by a low proliferation index (sensitivity) was 39% (13/33), whereas the proportion of non-responders with an intermediate/high index was 100% (7/7).

Among the patients with tumour recurrences, one patient with a low proliferative index responded to treatment compared with 4/8 patients with an intermediate/high proliferation rate ($p = 0.34$). The patient with the low proliferation index was still in CR together with one of the other patients.

A combined index versus tumour response

The seven non-responders all had a drug sensitivity index ≥ 1.5 and an intermediate/high proliferation index (Table 4). The specificity of this index combination was thus 100%. Among the 33 responders, 9 had this index combination for sensitivity of response of 73% (24/33).

Drug sensitivity and proliferation indices vs. survival

For overall survival, tumour CR provided strong prognostic information (Table 5). A low proliferation index was also significantly associated with longer survival, whereas the drug sensitivity index had no prognostic value. The combination of a high drug sensitivity index and intermediate/high proliferation (combined index) was no more informative than the proliferation index alone. However, none of the patients with a high drug sensitivity index (≥ 2.7) together with an intermediate/high proliferation

Table 4

Number of clinical responders (complete and partial) and non-responders according to drug sensitivity and proliferation index

	Drug Sensitivity Index				
	< 1.5		≥ 1.5		
	Proliferation Index		Proliferation Index		
	Low	Int./High	Low	Int./High	Total
Responders	8	11	5	9	33
Non-responders	0	0	0	7	7

Significant differences between responders and non-responders according to differences in drug sensitivity and proliferation index were $p = 0.006$ and $p = 0.04$, respectively.
Abbreviations: Int./High = intermediate or high.

Table 5

Hazard ratios (HR) for overall survival according to a univariate Cox's proportional hazards model. P-values below 0.10 are specified

Variable	Cut-off	n	e	HR	p-value
Treatment response	CR	23	8	1.0	
	Not CR	17	16	6.0	0.0001
Disease status	Primary	31	17	1.0	
	Recurrence	9	7	2.2	0.08
Drug sensitivity index	<1.5	19	11	1.0	
	≥ 1.5	21	13	1.3	n.s.
Proliferation index	Low	13	5	1.0	
	Int./High	27	19	2.8	0.04
Combined index	all other	24	12	1.0	
	≥ 1.5 + int/high	16	12	2.3	0.04
Stage	I–II	15	7	1.0	
	III–IV	25	17	1.8	n.s.

Abbreviations: CR = complete response; Int./High = intermediate or high; n = number of patients; e = events; HR = hazard ratio; n.s. = not significant.

rate (6 patients) was a long-term survivor (not shown). Analysis regarding progression-free survival showed essentially identical results (not shown).

Four patients were treated on the basis of the FMCA results. Three of these patients went into CR; two of whom later had tumour recurrences and died while the other patient is still alive. The FMCA and proliferation indices for these patients were 1.3 and intermediate, 3.0 and high and 1.0 and low, respectively. One patient with progressive disease and who died had an FMCA index of 3.0 with an intermediate proliferation index.

DISCUSSION

During the past decade, several non-clonogenic short-term ex vivo cytotoxicity assays with both cytotoxic and proliferative endpoints have shown promising results, comparable with those of clonogenic assays (22, 23). Overall, these ex vivo assays have shown similar predictive capabilities for clinical response, with sensitivities of 70–95% and specificities of 40–80% (22, 23). Some assays also show good correlation with patient survival (22). Furthermore, assay sensitivity or specificity does not differ with respect to patient treatment status (23). Thus, ex vivo assays seem adequately to mirror drug sensitivity and could be useful tools in the optimization of the use and development of cytotoxic drugs.

In the present study we investigated the ability of the FMCA to predict clinical outcome in aggressive NHL. Overall, the drug sensitivity data generated in the FMCA seemed to reflect the clinical outcome. Thus, for pivotal drugs in the treatment of aggressive NHL, tumour samples from responding patients seemed more sensitive than those from non-responders, although the differences were often of borderline significance. For some drugs, cell survival was similar for both groups, whereas for cisplatin there was a tendency toward a paradoxical inverse relationship between drug sensitivity ex vivo and clinical response. The reason for the latter observation is not clear but might indicate the presence of collateral sensitivity in aggressive NHL, i.e. increased sensitivity to a specific drug upon occurrence of resistance to other drugs (24). However, these results have to be interpreted with caution, since the group of non-responders only included seven cases.

Furthermore, there was a significant association between drug resistance profile ex vivo and clinical response at the individual patient level. However, whereas the ability to identify non-responders was high (specificity 100%), the ability to identify responders (sensitivity) was less impressive (58%). This contrasts with previous studies using FMCA as well as other short-term assay systems for which sensitivities and specificities in the range of 70–95% and 40–80%, respectively, were typically reported (22, 23). This performance has been attributed to various factors, including the suprapharmacological concentrations used as well as technical and biological factors inherent in the assay (25). However, the selection of cut-off value will also affect performance. Indeed, by changing the drug sensitivity index cut-off value to 2.7 instead of 1.5, the sensitivity rose to 82% but the specificity dropped to 43%. This is very similar to what has been reported previously with FMCA in haematological malignancies such as in acute lymphoblastic leukaemia, acute myeloid leukaemia and NHL of mainly low-grade phenotypes (17, 26–29). In clinical practice it may nevertheless be preferable to maximize the sensitivity at the expense of specificity since the probability of excluding potentially active treatment should be minimized.

Owing to the relatively large number of drugs used in the treatment of the patients, we used a drug sensitivity index as the measure of drug resistance. In doing so, all drugs in the panel influence the final score equally, irrespective of their pre-test probability of response. Pre-test

probability for specific drugs can be estimated from phase II clinical trials of single agents and theoretically may improve assay performance (26). However, there is a lack of good phase II documentation for many drugs used in NHL treatment and therapy is also mostly given as multi-agent combinations, making calibration of the ex vivo–in vivo correlation more difficult to perform. Nevertheless, both drug-specific calibration, implementing the pre-test probability of response, and the use of drug combinations should be considered in future studies with chemoresistance assays in aggressive NHL.

The results support the notion that the tumour response in vivo is due to other factors than intrinsic chemosensitivity as measured ex vivo alone. A specific feature of aggressive NHL is the wide range of tumour cell proliferation, which in vivo could lead to a repopulation between the courses of chemotherapy. Another potential effect of high proliferation rates is poor vascularization, which makes it difficult for the drugs to reach all the tumour cells (11, 30).

Head and neck cancers also generally have a rapid proliferation, which may be of prognostic importance (31, 32). In this context it is interesting to note that also in head and neck cancer, ex vivo drug-resistance testing was shown to give high specificity and low sensitivity (23). Furthermore, in both NHL and head–neck cancer, regrowth resistance has been implicated as an important determinant for response to chemotherapy (33).

Proliferation has prognostic value in NHL (7–10) and this was also observed in the present study. Like the drug sensitivity index, specificity was high (100%), whereas the sensitivity was low (39%). However, by combining the proliferation and the drug sensitivity indices, the sensitivity for tumour response increased to 73% at retained 100% specificity, indicating the complementary nature of these indices.

It is interesting to note that in aggressive NHL, tumour cell drug sensitivity assessed ex vivo was predictive of tumour response but not survival, whereas cellular proliferation also predicted the long-term outcome. From a tumour biology point of view these findings seem logical; the short-term effect of chemotherapy is clearly related to the drug sensitivity of the tumour cells, whereas additional factors, notably the capacity of remaining tumour cells to regrow, will be decisive for tumour recurrence and, thus, long-term survival. Since the FMCA measures the effect of a cytotoxic drug in the total population of tumour cells, it will not be able to reflect tumour microheterogeneity, e.g. differences in proliferative capacity in surviving cells (23). Thus, the inability of the FMCA to predict survival in aggressive NHL is not surprising. However, since total cell kill assays similar to the FMCA have been found to predict survival (34), it seems reasonable to conclude that the intrinsic tumour cell drug sensitivity is relatively more important in other tumour types. Thus, depending on tumour type, it might be necessary to add to drug sensitivity, information on regrowth capacity to predict the long-term outcome.

In summary, the present study indicates that the FMCA, in contrast to the situation in other tumour types, needs to be complemented by information on tumour cell proliferation to provide strong predictive information or tumour response in aggressive NHL. Furthermore, only cellular proliferation seems to be predictive of the long-term outcome. Although this patient material was small and heterogeneous and the analyses carried out retrospectively, the results warrant further attention in prospective studies exploring laboratory predictions of treatment outcome in NHL.

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