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Development and Characterisation of a Cyclophosphamide Resistant Variant of the BNML Rat Model for Acute Myelocytic Leukaemia

Anton C.M. Martens, Cees J. de Groot and Anton Hagenbeek

A cyclophosphamide resistant subline (BNML/CPR) was developed *in vivo* in the BN rat acute myelocytic leukaemia (BNML) model. Full resistance was achieved after *in vivo* exposure of leukaemic animals to cyclophosphamide with, in total, 15 intraperitoneal injections of 100 mg/kg. The CPR line was cross-resistant to ifosfamide, but less so to mafosfamide. Continuous transplantation of the BNML/CPR line without a cyclophosphamide selection pressure resulted in the emergence of a subline (BNML/CPR>S) whose sensitivity to cyclophosphamide was similar to that of the parent BNML/S line. Both in the BNML parent line and in the BNML/CPR>S line, a 2p+ marker chromosome was present, whereas a 2p+q+ marker chromosome was characteristic for the BNML/CPR line. The mechanism of cyclophosphamide resistance can now be investigated in the BNML model at the DNA, at the mRNA and at the protein level.
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INTRODUCTION

RESISTANCE TO the cytostatic drugs for human cancer is still the major cause of failure to cure. One of the most widely used drugs is cyclophosphamide, which is incorporated in many treatment protocols for haematological malignancies, carcinomas and sarcomas [1].

Cyclophosphamide first has to be metabolised by the liver to yield the activated 4-hydroxy form. The next metabolite is aldophosphamide, which is metabolised to the cytotoxic phosphoramide mustard (PM) or to the deactivated carboxyphosphamide [1]. In deactivation, aldehyde dehydrogenase (ALDH) has an important role [2, 3].

Two enzyme systems may be involved in cyclophosphamide resistance—i.e. ALDH [4, 5] and glutathione-S-transferase and glutathione-dependent enzymes [6]. Increased intracellular ALDH has been correlated with reduced sensitivity to cyclophosphamide as well as to the activated metabolites [7]. Pretreatment with inhibitors of ALDH such as disulphiram resulted in restoration of the sensitivity for CP of CP resistant cell lines [8, 9]. However, others have reported that resistance to cyclophosphamide and to nitrogen mustards is correlated with increased levels of glutathione, glutathione-S-transferase or other glutathione-dependent enzymes [10, 11]. Other mechanisms, such as changes in drug transport and/or increased repair of cyclophosphamide-induced DNA lesions, may be involved. A prerequisite for studying resistance is the availability of a cyclophosphamide resistant cell line with well-defined characteristics. There are few cyclophosphamide-resistant animal tumour models available [6, 10, 12–15] and few *in vitro* cell lines of human origin [16–18].

In the rat, a model for acute myelocytic leukaemia (BNML) has proven suitable for preclinical research [19].

Here we describe the *in vivo* development of a cyclophosphamide resistant cell line from the BNML model (BNML/CPR),

Correspondence to A.C.M. Martens.
A.C.M. Martens and A. Hagenbeek are at the Institute for Applied Radiobiology and Immunology/TNO Lange Kleiweg 151, 2280 HV Rijswijk; and C.J. de Groot is at, and A. Hagenbeek is also at the Dr Daniel den Hoed Cancer Center, Rotterdam, The Netherlands.
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cross-resistance to cyclophosphamide related drugs and the loss of resistance on continuous passaging without a cyclophosphamide selection pressure. The chromosomal aberrations involved are documented.

MATERIALS AND METHODS

Rats

The rats were from the SPF inbred Brown Norway (BN) strain BNBi/Rij, produced in the Rijswijk breeding colony. Male rats between 13 and 16 weeks of age were used (mean body weight 260 g).

Leukaemia model

The BNML model has been described [19]. Briefly, after intravenous cellular transfer, the leukaemia shows a reproducible growth pattern. Some of its major characteristics are: (1) a slow growth rate, (2) severe suppression of normal haemopoiesis due to an absolute decrease in the number of haemopoietic stem cells (CFU-S, colony-forming units spleen), (3) the presence of clonogenic leukaemic cells (*in vivo*, LCFU-S, leukaemic colony-forming units spleen; *in vitro*, colony assays), and (4) response to chemotherapy as in human acute myelocytic leukaemia.

Preparation of cell suspensions

Cell suspensions were made from the spleens of leukaemic animals. Part of the spleen was minced with scissors and gently pressed through a nylon sieve with a spatula to obtain a monocellular suspension in Hanks' HEPES buffered balanced salt solution. The cell concentration was counted after dilution and staining with Türks' solution, in a Bürker haemocytometer. The cell suspension was adjusted to the required concentration for injection into animals.

Spleen colony assay for clonogenic leukaemic cells (LCFU-S)

Three groups of 7 normal rats each were injected with graded numbers of cells chosen such that 5–30 colonies would be visible on the recipient's spleen after killing the animals 19–20 days later and fixing the spleens in Telleyesniczky's solution containing 70% ethanol, 20% formaldehyde and 10% acetic acid.

Each colony visible on the surface of the spleen is assumed to have been derived from 1 leukaemic cell that originally lodged in the spleen. For each injected cell dose, the mean corresponding spleen colony count was calculated. The three mean values were averaged to obtain the LCFU-S content.

Survival assay

This assay was based on the observed linear relation in the BNML model between the injected number of cells and survival [19]. From this relation, the effect of antileukaemic treatment could be deduced by measuring the prolongation of survival—i.e. 4 days' increase in lifespan (ILS) corresponds with a 10 fold reduction in tumour load, referred to as 1 log cell kill (LCK). The surviving fraction obtained with a certain drug treatment was calculated with the formula: surviving fraction = $10^{-ILS/4}$.

Treatment with cytostatic drugs

Cyclophosphamide and ifosfamide were routinely given intraperitoneally. Mafosfamide was injected intravenously. The drugs were kindly provided by Dr P. Hilgard and Dr J. Pohl from Asta Werke A.G., Bielefeld, Germany.

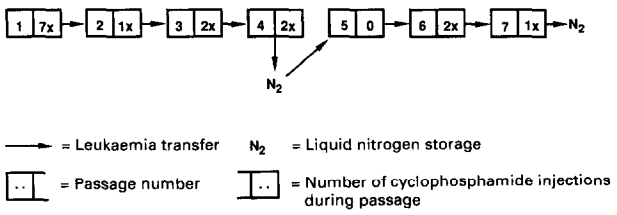

Fig. 1. Diagram of development of BNML/CPR line.

RESULTS

Induction of cyclophosphamide resistance in the BNML model

To mimic the clinical situation, in which human tumour cells are repeatedly exposed to cyclophosphamide, the following *in vivo* treatment strategy was chosen to induce resistance in cells from the BNML model. Cyclophosphamide 100mg/kg injected at day 13 after intravenous transfer of 10^7 BNML cells induces a 20 day ILS of the leukaemic animals, corresponding to a 5 LCK [20]. Such a treatment also affects normal haemopoiesis; however the reduction in the haemopoietic stem cell population is limited to 1–2 logs [21]. This implies that when animals are treated every 2 weeks, normal haemopoiesis would be fully restored when the subsequent treatment with cyclophosphamide is given. After each exposure to cyclophosphamide a group of rats was separated for assessment of the effect of the last dose.

During the first leukaemia passage the animals received 7 injections of cyclophosphamide. 2 weeks after the last injection the cell line was transferred to new recipients. These rats were treated with cyclophosphamide 100 mg/kg 2 weeks later (Fig. 1). When the subsequent injection of cyclophosphamide was to be given, some of the treated animals had already died of recurrent leukaemia, indicating that survival after treatment was shortening and possibly that resistance was emerging. Leukaemic spleen cells were therefore transferred to new recipients and cyclophosphamide treatment and transplantation intervals were shortened. During the next two passages (number 3 and 4) the rats were treated twice with cyclophosphamide 100 mg/kg. Passage 4 (so far treated 12 times with drug, stage 4–12) was analysed for response to cyclophosphamide treatment and the regrowth characteristics of the surviving cells were compared with that of the sensitive parent line BNML/S. From passage 4–12 cells were frozen and a stock was stored in liquid nitrogen. The cell-dose survival experiment is shown in Fig. 2. The rate of regrowth at this stage was not significantly different from the parent line and the method of calculating tumour load reduction (a 4 day ILS equals 1 LCK) could still be used. The ILS of stage 4–12 after cyclophosphamide treatment was 8 days (2 LCK; Table 1). Such a treatment generally results in the BNML/S line in an increase in survival time of 20–24 days corresponding to 5–6 LCK. This means that at this stage a 3–4 log less reduction in tumour load was achieved with cyclophosphamide treatment.

This reduced effect of cyclophosphamide was confirmed by measuring changes in the spleen weight when the leukaemic cells were regrowing after drug treatment. Extrapolation of the spleen weight curves for BNML/CPR stage 4–12 to the time of treatment yields a factor of 10 (1 log) reduction in tumour-load compared with the control spleen weight curve (Fig. 3). Extrapolation of the spleen regrowth curve of the BNML/S line after cyclophosphamide treatment indicated a 4 log difference compared with the spleen weight curve of BNML/S controls. This was in agreement with the survival data. Full resistance,

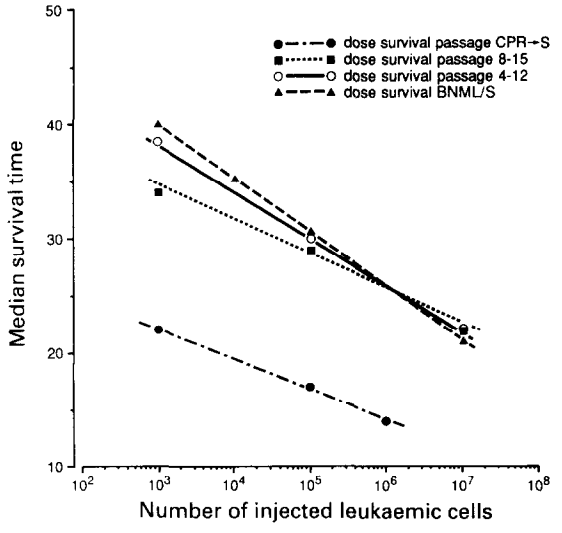

Fig. 2. Survival curves for various BNML variants at different stages during acquisition and loss of cyclophosphamide resistance.

however, was not yet achieved. Therefore we decided to continue cyclophosphamide exposure.

Cells were thawed from the frozen stock and to allow re-adaption to *in vivo* growth, this passage (number 5) was not treated with cyclophosphamide. Passage 6 was treated twice with drug and passage 7 once. The effect on the survival time was monitored (Table 1). When passage 7–15 was transplanted (stage 8) and treated with cyclophosphamide the increase in median survival time of 0.5 day indicated that full resistance was achieved. Passage 7 (stage 7–15) was frozen and kept in a large stock in liquid nitrogen. For new experiments, cells from this stock were thawed, passaged once (yielding passage 8–15) and used. This line is referred to as "the CPR line" and was characterised in detail. The cell-dose survival curve indicated that the growth rate of the CPR line had increased somewhat: a

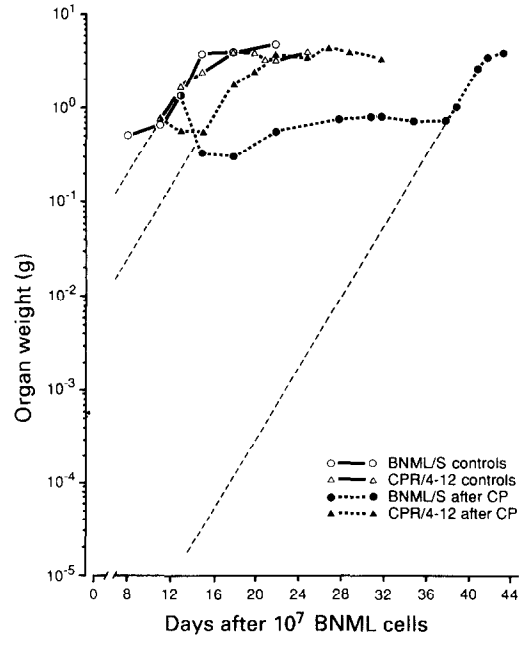

Fig. 3. Regrowth of leukaemic cells surviving cyclophosphamide treatment measured by spleen weight.

Table 1. Decreasing effectiveness of cyclophosphamide during repeated passages of BNML under cyclophosphamide selection pressure

Passage no.	Day of treatment	Cumulative no. of injections	Observed ILS (days)
1	13 (7×)	7	20
2	13	8	8
3	12 (and 23)	10	NT
4	8 (and 19)	12	8
5	No drug	12	—
6	8 (and 22)	14	2
7	8	15	2
8	12	16	0.5

NT = not tested.
ILS = increase in lifespan.

factor of 10 less injected cells resulted in an increase in the survival time of 3 days; in comparison, this was 4 days for the BNML/S as well for the CPR/stage 4–12 (Fig. 3).

The effect of treatment of the CPR line with cyclophosphamide doses of 100, 140, 160 and 200 mg/kg is shown in Fig. 4. The surviving fraction was assessed by LCFU-S, which is more sensitive than the survival assay for surviving fractions between 1 and 10^{-2} (between 0 and 2 LCK). Treatment with 100 mg/kg resulted in a surviving fraction of 6.8×10^{-1} (Fig. 4). The higher doses—i.e. 140, 160 and 200 mg/kg—resulted in a surviving fraction of LCFU-S of 2.4×10^{-1}, 1.3×10^{-1} and 0.6×10^{-2}, respectively. The dose-effect curve for the CPR line (LCFU-S) was compared with the dose-effect curve for the BNML/S line for which the survival assay was used as an index

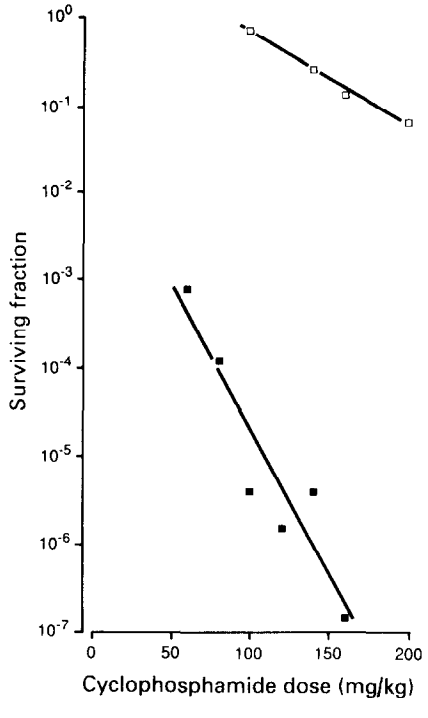

Fig. 4. Comparison of surviving fraction of BNML/S (with survival assay) and BNML/CPR (with LCFU-S assay) after treatment with cyclophosphamide. Open squares = BNML/CPR, closed squares = BNML/S.

Table 2. Cross-resistance pattern of the BNML/CPR compared with BNML/S line after treatment with various cytostatic agents

Drug	BNML line	Exp. no.	Day of treatment	ILS based on MST (days)	Cross resistance*
Mafosfamide (154 mg/kg)	CPR	2	13	3.5–4.5	+/−
	S	2	13	11–16.5	
Ifosfamide (200 mg/kg)	CPR	1	13	0	+
	S	1	13	16.5	
Cyclophosphamide (100 mg/kg)	CPR	7	12	0	+
	S	7	13	20–24	

*+/− = partial and + = complete cross-resistance.
MST = median survival time.

to measure the surviving fraction (Fig. 4). For the BNML/S the fraction ranged between 10^{-3} and 10^{-7}, while for the BNML/CPR the values were between 6.8×10^{-1} and 6×10^{-2} for the drug dosages used.

Cross-resistance of BNML/CPR

Cross-resistance of the BNML/CPR to treatment with ifosfamide and mafosfamide was compared with the response of the BNML/S parent line. The doses of ifosfamide and mafosfamide were chosen such that an increase in survival time close to that after cyclophosphamide at 100 mg/kg intraperitoneally would be obtained. Treatment of the CPR line with ifosfamide 200 mg/kg intraperitoneally did not result in an increase in survival time compared with untreated controls (Table 2). Mafosfamide 154 mg/kg intravenously, however, resulted in a 3.5 and 4.5 days ILS (two separate experiments), indicating that 90% of the leukaemic cells were eliminated (1CK).

Generation of a cyclophosphamide sensitive subclone from BNML/CPR

The stability of cyclophosphamide resistance in the CPR line was studied by continuously transplanting the CPR line into new recipients. The animals were not treated with cyclophosphamide—i.e. there was no selection pressure for the resistant population. The survival times were recorded (Table 3A). Some intermediate passages were tested for the degree of sustained resistance by treatment with cyclophosphamide at 100 mg/kg. When passage numbers 5, 8 and 15 were treated, there was no increase in survival time compared with the control group (Table 3A/B). However, from passage 16 onwards, a more rapidly proliferating subpopulation emerged, indicated by shorter survival times in the controls. Concurrently, the resistance to cyclophosphamide gradually disappeared during the subsequent passages, indicated by the fact that drug treatment of animals at this stage resulted in an increase in survival time.

During passage 19 very low numbers of leukaemic cells were injected in an attempt to select the emerging, more rapidly growing, presumably cyclophosphamide sensitive clone. Indeed, treatment of the next passages with cyclophosphamide indicated that this approach was successful. A highly sensitive cell line, named BNML/CPR>S, was derived after passage 21. Animals from passage 22 could be cured after treatment on day 7. Treatment of passage 23 at day 9 (corresponding with a higher tumour load) resulted in an ILS of 21–29 days. Because a dose-survival experiment indicated that a 10 fold lower number of cells corresponded with a 3 days' ILS (Fig. 3), we could calculate that the observed 24 days ILS corresponded to an 8 LCK. At this stage, we concluded that the cyclophosphamide sensitive line BNML/CPR>S had emerged. A large stock of passage 23 was stored in liquid nitrogen.

Table 3. Stability of the BNML/CPR line on continuous passaging and emergence of BNML/CPR>S line

Passage no.	Cells (^{10}log) on day:	Cyclophosphamide on day:	Survival (days)	ILS
(A) Continuous passaging				
1–14	7	—	16–21	—
15	7	—	12–18	—
16	7	—	11–24	—
17	7	—	12	—
18	6	—	12	—
19	2	—	24–32	—
20	6	—	14–16	—
21	6	—	14	—
22	6	—	16–20	—
23	6	—	13–14	—
(B) Cyclophosphamide treatment of various passages				
5*	7	8	19*	3
8*	7	12	17–19*	1–3
15	7	7	18–20	2–8
17	7	7	17–25	5–13
18	6	7	29–30	13–14
20	6	7	31–73	1–58
21	6	7	57–60	43–46
22	6	7	>100	cured
23	6	9	34–43	21–29

*Compare with control survival passage numbers 1–14 in (A).
Number of animals per group varied between 2 and 8.
ILS = compared to controls (A).

Cytogenetic analysis of cell lines

Cytogenetic analysis of the BNML/S, the CPR and the CPR>S lines revealed that the BNML specific 2p+ chromosome [22] was replaced by a 2p+q+ marker chromosome in the CPR line (Table 4). In the CPR>S line, however, a 6p+, inv(7) and an 18q− marker chromosome were found besides all BNML/S associated aberrations.

DISCUSSION

Our objective was the *in vivo* development of a cyclophosphamide resistant variant of the BNML model. A resistant line was achieved, thereby offering a model for studying cyclophosphamide resistance at the enzymatic, chromosomal and nucleic acid level.

During the first phase, rats received repeated injections of cyclophosphamide every 2 weeks. Except for the first injection,

Table 4. Cytogenetic analysis of BNML/S, CPR line and CPR>S

Cell line	Chromosome aberrations								
BNML/S	1p+	2p+			8q+	−9	12q−		20q+
CPR	1p+		2p+q+		8q+	−9	12q−		20q+
CPR>S	1p+	2p+		6p+inv(7)	8q+	−9	12q−	18q−	20q+

the tumour load was not known at the time of treatment. However, after transfer of the leukaemic cells to new recipients and subsequent treatment with cyclophosphamide, survival time was shortened. The cell-dose survival relation showed that at this stage the growth rate had not changed (Fig. 2), which implied that a resistant subclone was emerging. When an intermediate passage (i.e. 4–12) (Table 1) was analysed, the extrapolation of the regrowth curve of leukaemic cells measured by spleen weight indicated a surviving fraction of 10% after cyclophosphamide treatment (Fig. 3). This would correlate with 10% of the cells belonging to the CPR clone and 90% of the cells being of the BNML/S type. It also explains that, with only a few more passages and exposures to cyclophosphamide, full resistance was achieved. On injection of CPR cells into healthy recipient rats, which had not received cyclophosphamide before, full resistance of the leukaemic cells to injected cyclophosphamide was observed. This rules out changes in the host tissues, but points to intrinsic changes in the leukaemic cells being responsible for the observed resistance.

There was a large difference in sensitivity for cyclophosphamide between the BNML/S and the BNML/CPR line. At doses that are curative in a fraction of BNML/S leukaemic animals—e.g. cyclophosphamide 200 mg/kg [21] correlating with 9–10 LCK—6% of the CPR cells survive (Fig. 3). This is only slightly more than 1 LCK. The difference in the surviving fraction at this dose was 8 to 9 LCK. The cell-dose survival relation (Fig. 2) indicated that the growth rate of the CPR line was increased compared with that in BNML/S. A factor of 10 less cells corresponded to a 3 day ILS compared with 4 days for the parent BNML/S line.

Cross-resistance was observed for ifosfamide, which was not surprising since this drug is closely related to cyclophosphamide and follows the same metabolic pathway. A lower degree of cross-resistance was observed for mafosfamide. About 90% of the CPR cells are killed by mafosfamide. This drug, once dissolved, yields the activated 4-hydroxy metabolite. This may lead to higher plasma levels for mafosfamide upon intravenous injection, compared to a more slowly rising and ultimately lower serum level of 4-hydroxycyclophosphamide for cyclophosphamide itself [23].

The continuous transfer to new recipients of the CPR line without a cyclophosphamide selection pressure showed stable growth until the 15th passage (Table 3A), which is in agreement with observations of other investigators [24]. However, the survival time of the control group started to decrease, indicating the emergence of a more rapidly growing subclone. At the same time, resistance to cyclophosphamide rapidly decreased. Passage 19 was a successful attempt of "*in vivo* cloning" of the emerging sensitive CPR>S variant. During the next passages it became apparent that a cell line fully sensitive again for cyclophosphamide had emerged (Table 3B). The dose-survival curve of this CPR>S line (Fig. 2) indicated that certain cell kinetic indices (e.g. growth fraction, cell cycle variables and cell loss fraction) were different. The origin of the CPR>S cell line is not clear. There are three periods during which it may have developed. It might have been present as a subclone in the (original) BNML parent line, it may have emerged during the resistance induction period with the repeated cyclophosphamide treatments or during the period of serial passaging without drug treatment. Its rapid (re)growth characteristics (Fig. 2) may have prevented its extinction during the resistance induction treatment. This rapid growth is also responsible for overgrowing the CPR line during the continuous passaging without cyclophosphamide treatment (Table 3). The three cell lines BNML, CPR and CPR>S were cytogenetically distinct from each other (Table 4). For studies of cyclophosphamide resistance mechanism, a frozen stock is available which is never carried on for more than two passages. Therefore loss of the resistant clone is not an issue: up to 8 passages without cyclophosphamide were allowed (Table 3B) with no change in the resistance to drug treatment.

Gene amplification of enzyme systems involved in detoxification processes has been found to be involved in the development of drug resistance [25, 26] and is often associated with chromosomal aberrations. For this reason the chromosome composition of the various BNML variants was studied. The CPR line is characterised by only one chromosomal aberration different from the BNML/S line—i.e. a 2p+q+ marker chromosome in the CPR line instead of the typical 2p+ marker chromosome in case of the BNML/S [26]. This 2p+q+ marker chromosome was not present in the BNML/CPR>S line. Because additional aberrant chromosomes were found in the CPR>S variant, the involvement of other chromosomes than the 2p+q+ in cyclophosphamide resistance cannot be excluded. Whether this chromosomal change is related to the gain and loss of the resistance in the BNML model, thereby forming its genetic basis, is an attractive hypothesis but remains to be investigated. Although the CPR>S line was very sensitive, its high proliferation rate might have prevented its extinction during the selection of the CPR line and, as a consequence, it may have been present as a subclone continuously. The CPR/S related additional chromosomal aberrations probably developed during the initial resistance induction phase. During the repeated transplantation of the cell line without a cyclophosphamide pressure, its rapid proliferation rate resulted in a growth advantage over the CPR line.

That the basis for cyclophosphamide resistance can be associated with amplification of the genes coding for certain detoxifying enzymes (e.g. glutathione-S-transferase), increased levels of mRNA, increased stability of the mRNA or the enzymes, has been shown for nitrogen mustard resistant cell lines [22]. For studying genes coding for cyclophosphamide resistance at the DNA level, cDNA probes are needed for the relevant enzymes that are involved in the de-activation metabolism of the drug. The availability of a cDNA probe for (human) ALDH1 [27] will allow the exploration of a possible role of this enzyme in the cyclophosphamide resistance mechanism in the BNML/CPR model. cDNA probes are also available for human and rat glutathione-S-transferase ρ and π class mRNAs [28, 29]. *In situ* hybridisation on metaphase spreads of normal BN, BNML/S, CPR and CPR>S cells or spot-blot hybridisation of flow cytometry sorted chromosomes will allow the localisation of the genes encoding for these enzymes on the chromosomes from normal rats as well as from the leukaemic variants.

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Ethical Issues in Psychosocial Research among Patients with Cancer

Anne Eardley, Alan Cribb and Laura Pendleton

The ethical implications of psychosocial research among patients with cancer are discussed. Two key issues were identified: obtaining informed consent and the impact of participating in research. Barriers to obtaining genuinely informed consent are described, as well as the costs and benefits of participation in research. Recommendations are made for the conduct of future research, relating to the removal of barriers to informed consent and monitoring the impact of the research process on its subjects.
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