Eur J Cancer, Vol. 29A, No. 1, pp. 116–122, 1993.
Printed in Great Britain

0964–1947/93 $5.00 + 0.00

Metabolic Response of AH13r Rat Tumours to Cyclophosphamide as Monitored by pO_2 and pH Semi-microelectrodes

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The composition of the microenvironment has an important influence on the cellular response to cytotoxic agents. Using pH and pO_2 semi-microelectrodes, we have monitored metabolic changes in AH13r rat tumours as a function of time after subcurative chemotherapy. Prior to therapy, tumours contained large areas considered hypoxic (mean $pO_2 \sim 4$ mmHg) and are characterised by a marked accumulation of acidic metabolites (mean pH 6.65). Administration of cyclophosphamide (40 mg/kg body weight) resulted in tumour regression to 15% of pretreatment volumes and a growth delay of 12 days. Concomitant with volume reduction, tumours became reoxygenated (mean $pO_2 \sim 7$ mmHg), with maximum values being reached within 2–4 days, paralleled by a shift of pH to more alkaline values (0.17 U on average). These changes coincided with the development of subtotal necrosis. During early tumour regrowth, the pH and pO_2 histograms returned to control values. These data corroborate and extend the results of previous studies in which noninvasive techniques had been applied for the monitoring of treatment-induced metabolic changes in malignant tumours *in vivo*. In addition, these results support the notion that the effectiveness of anticancer therapy might be improved by selecting and scheduling therapeutic agents in consideration of physiological changes caused by preceding courses of treatment.
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INTRODUCTION

THE RESPONSE of malignant cells to drug or radiation treatment *in vivo* depends on a complex interplay of various determinants. Of primary importance is, of course, the intrinsic sensitivity of individual cells to the therapeutic agents employed—as determined, for example, by their proliferative status as well as by bidirectional transmembrane drug transport, drug biotransformation and DNA repair. There are, however, additional determinants which have the potential to modulate the cytotoxic effects of anti-cancer agents. Among these, the composition of the cellular microenvironment deserves particular attention. Indeed, microenvironmental determinants of cytotoxicity may under certain conditions have an even greater impact on the outcome of treatment than the inherent characteristics of the target cells [1–5]. Physiological factors modulate cytotoxic effects either by direct (physical/chemical) interaction with a given agent as, for example, in the oxygen-dependent generation of toxic radicals by drugs, or indirectly by influencing cellular functions relevant to drug or radiation cytotoxicity [6–9]. Examples of the latter mechanisms include pH-sensitive drug transport and bioreductive drug activation. The selection and scheduling of therapeutic agents for combined modality treatment thus require consideration of the target cells' physiological status and its treatment-induced changes.

We have used a rat tumour model to investigate whether chemotherapy causes changes in two major microenvironmental determinants of drug and radiation cytotoxicity, pH and pO_2. In particular, the question has been addressed whether there are time intervals following drug therapy which would provide improved microenvironmental conditions for the sequential administration of various classes of cytotoxic agents. pO_2 and pH were chosen as parameters for two reasons: first, oxygen and lactic acid—the most important determinant of intratumoral pH [10]—are major constituents of tumour metabolism and thus prone to be affected by cytoreduction. Second, changes in both pH and pO_2, apart from their effects on DNA replication, cell cycle progression and cellular metastatic potential, are known to modulate the effects of various modalities for cancer treatment including irradiation, chemotherapeutic agents and hyperthermia [2, 3, 8,11–19].

In previous studies, the physiological response of tumours to cytotoxic treatment had been monitored mainly by indirect techniques, for example by radiobiological assays or non-invasively by nuclear magnetic resonance (NMR) spectroscopy or positron emission tomography (PET) [20, 21]. Only few attempts have been made to correlate the results of these investigations with direct (invasive) measurements of pH or pO_2 [22, 23]. Here we report the results of *simultaneous* direct measurements of pH and pO_2 in AH13r tumours, using hydrogen ion- and oxygen-sensitive semi-microelectrodes prior to, and at various intervals following cyclophosphamide (CP) administration.

MATERIALS AND METHODS

Animals and tumours

Adult female Sprague–Dawley rats weighing 250–300 g (Zentralinstitut für Versuchstierzucht, Hannover, Germany) were kept on a 12 h light–dark cycle with free access to a

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Revised and accepted 23 June 1992.

standard diet (Altromin, Lage, Germany) and water. A CP-sensitive subline of the rat Yoshida sarcoma, AH13r, was kindly provided by Dr J. Pohl (Asta Pharma, Bielefeld, Germany). At 2-week intervals, pieces of tumour tissue (~ 2 mm^3) were serially transplanted by trocar subcutaneously into both flanks of the rats. Tumour size was estimated by measuring two external diameters with calipers and calculating tumour volume (V) by the formula $V = 1/2ab^2$, where a is the longest and b the shortest diameter. Tumour growth delay (GD) following CP administration was assessed by measuring the time (days) required for the treated tumours to reach twice the pretreatment size (T) minus the time required for the doubling of tumour volume in control animals (C), i.e. GD = T − C. Cell kill was estimated as described by Evelhoch *et al.* [24]. One day before drug treatment, a central venous catheter was implanted as previously described [25]. All investigations were performed according to legislative regulations and institutional guidelines for animal experimentation.

Drug

CP (Asta Pharma, Frankfurt, Germany) was dissolved in sterile water. One third of the total dose of 40 mg/kg body weight was injected intravenously. Beginning immediately after drug administration, the remaining dose was continuously infused over a period of 6 h. Control animals received 0.9% (w/v) saline solution at the same rate (1 ml/h). Previous experiments (not shown) had demonstrated that this schedule resulted in a longer growth delay than single or split-dose injections of CP.

pH *and* pO_2 *measurements with the aid of semi-microelectrodes*

Tumour-bearing rats were anaesthetised with sodium pentobarbital (Nembutal®, 30 μg/g body weight intraperitoneally; Abbott, Bad Segeberg, Germany) and immobilised on a heating pad. Rectal and tumour temperatures were recorded by thermocouples. Immediately prior to the measurements, the skin and fibrous tissue overlying a tumour surface area of ~ 10 mm^2 were carefully removed. Particular care was taken not to damage blood vessels on the tumour surface.

For pH measurements, combined glass electrodes incorporated in a 25 gauge bevelled steel needle were used (tip diameter, 0.5 mm; No. 802, Diamond General Inc., Ann Arbor, Michigan, USA). pH electrodes were connected to a type 616 electrometer (Keithley Instruments Inc., Cleveland, Ohio, USA) and calibrated at 37°C in physiological phosphate buffers (pH 5.0, 6.03, 6.83, 7.15 and 7.33). Electrode characteristics were as follows: slope, 58–60 mV/pH unit; response time (90%), 10–20 sec; drift, < 0.21 mV/h. All calibrations and measurements were performed in an electrically shielded cage using mechanically driven remote control micromanipulators as previously described [26]. Electrodes were continuously inserted to a depth of 5–10 mm (depending on tumour size) at a rate of 0.5 mm/min. To improve representative sampling, two electrodes were mounted in parallel (tip distance, 3–4 mm) and used simultaneously. For calculation of pH frequency distributions, single point pH values were obtained from the continuously recorded electrode signals at distances of 0.5 mm.

Tissue oxygenation was determined with an improved electrode system according to Weiss and Fleckenstein [27]. The application of this technique to measurements in malignant tumours has previously been described [28–30]. Steel shafted needle probes with a ground lancet-type tip (diameter, 350 μm) were used for the pO_2 measurements [27]. Integrated into the tips was a membranised, recessed gold microcathode of 12 μm diameter. A Ag/AgCl wire implanted subcutaneously into the dorsum of the animals served as anode in the polarographic circuit. The probes were inserted stepwise into the tissue by motor-driven micromanipulators. The steps consisted of a rapid foreward motion of 1 mm immediately followed by an equally rapid backward movement of 0.3 mm ("pilgrim step"). This type of movement was used in order to relax the mechanical pressure exerted by the tip of the probes during pO_2 measurements. Every 700 μm, at the end of each step, a single local pO_2 measurement was made after 1.4 s, taking into account the response time of the probes (< 500 ms). Electrode signals were processed by a pO_2 histograph (Sigma pO_2-Histograph/KIMOC, Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) and displayed online as pO_2 vs. time plots. Depending on tumour size, electrodes were inserted into individual tumours 3–5 times at different sites in order to record 60–100 single-point pO_2 values per tumour. The PO_2 probes were two-point calibrated in buffered 0.9% saline solution (pH 7.4) equilibrated under atmospheric pressure with air or N_2 and recalibrated after final withdrawal from each tumour. pO_2 readings were corrected for differences between calibration chamber temperature and tumour tissue temperature, respectively. The mean tumour temperature was 36.5 ± 0.5°C. The rectal temperature of anaesthetised animals was kept at 37 ± 0.5°C by infrared irradiation.

Statistical analysis

Differences in mean pH values between the various test groups were determined by Student's t-test. Statistical comparison between pO_2 frequency distributions was performed by the Mann–Whitney U-test. P values < 0.05 were judged to be of statistical significance.

Pathological studies

Rats were killed with ether immediately following electrode measurements. Then tumours were dissected and cut in a plane encompassing one or more electrode tracks. Gross pathology was recorded and tumour slices were fixed in 10% buffered formalin. Specimens were embedded in paraffin and 5 μm sections were stained with haematoxylin–eosin.

RESULTS

Effect of CP on growth and histological appearance of AH13r tumours

The volume doubling time of untreated AH13r tumours (mean size 0.9–11.7 cm^3) was ~ 2 days, similar to the regrowth rate of tumours following subcurative CP treatment (Fig. 1a). CP (40 mg/kg body weight) was administered intravenously over a period of 6 h (for details, see Materials and Methods) when tumours had grown to a mean volume of ~ 4.0 cm^3. Within 6 days after treatment, the declining growth curves reached their nadirs at ~ 0.7 cm^3. Thereafter, growth resumed and a tumour volume equal to pretreatment size was reached after ~ 12 days (Fig. 1a). From these data it can be estimated that in CP-treated tumours less than 2% of the tumour cells survived [24].

Tumours were dissected for histological examination at the same times as for physiological studies, i.e. at volumes of ~ 1.5 cm^3 (control group I) and ~ 4 cm^3 (control group II), as well as on days 1, 2, 4, 8 and 12 following CP administration (Figs 1, 2). In histological sections, tumours appeared as large islands of densely packed anaplastic cells separated by broad strands of intervening fibrous tissue and interspersed with

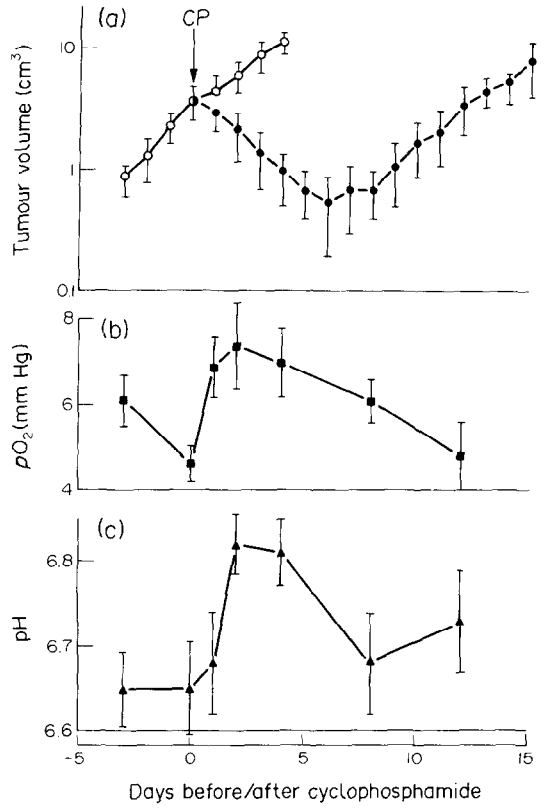

Fig. 1. Correlation of volume changes with physiological parameters in AH13r tumours prior to and following CP treatment (40 mg/kg body weight). Tumour-bearing animals were treated on day 0 (arrow) with saline (○) or 40 mg/kg CP (●, ■, ▲). Points, mean values of 8–10 tumours (SD) (a) or (SEM) (b, c). (a) Tumour volume; (b) oxygen partial pressure; (c) pH.

small areas of light haemorrhage, a pattern consistent with the macroscopic findings upon tumour dissection (Fig. 2a,b). Tumours of group I were essentially free of necrotic areas (Fig. 2a). However, at a tumour volume of ~ 4 cm^3, small foci of necrosis became apparent in central areas of the tumours. At this tumour size, the necrotic foci did not coalesce to involve more than an estimated 10% of the total volume (Fig. 2b). In early posttreatment tumours (day 2) necrotic changes became apparent (Fig. 2c) and on day 4, more than two thirds of the central tumour sections consisted of necrotic areas (Fig. 2d). Later on, tumours were repopulated from small and rapidly expanding pockets of surviving cells, a process first apparent on day 8 (Fig. 2e) and complete by day 12 (Fig. 2f). Marked infiltrations by monocytes or polymorphonuclear cells were not seen at any time.

Oxygenation and pH *in AH13r tumours following CP treatment*

The oxygen histogram of skeletal muscle comprises pO_2 values of 0–80 mmHg, with mean and median values of 31 and 29 mmHg, respectively (Fig. 3). Compared with this frequency distribution, AH13r tumours growing subcutaneously in the flanks of Sprague–Dawley rats appeared to be severely hypoxic, although marked regional heterogeneity was observed in individual tumours, with pO_2 values ranging from ~ 55 mmHg to readings indistinguishable from zero (Fig. 4a,b). The class of pO_2 values < 5 mmHg was found at frequencies of 75% immediately prior to chemotherapy and 80% in regrowing tumours of the same size (Fig. 4b,f). CP treatment resulted in a 60% increase in the mean pO_2 as compared with size-matched controls, a process becoming apparent on day 1 after drug administration ($P < 0.01$) and continuing until the end of tumour growth delay (day 8: mean pO_2, 6 mmHg; median pO_2, 4 mmHg) (Figs 1b, 4c–e). Reoxygenation was maximal on day 2 (mean pO_2, 7 mmHg; median pO_2, 5 mmHg; $P < 0.05$). At this time, the frequency of pO_2 readings < 5 mmHg was reduced to 57%, whereas tumour areas exhibiting pO_2 values of 5–10 mmHg had expanded from 16% (control group II) to 27% (Figs 1b, 4c). Although in regressing tumours single pO_2 readings as high as 60 mmHg were occasionally recorded, the overall oxygenation of AH13r tumours never approached that of skeletal muscle, as shown in pO_2 histograms (c) and (d) of Fig. 4 and the frequency distribution displayed in Fig. 3. During regrowth, the treatment-mediated increase in local pO_2 values gradually declined, and on day 12 the pO_2 histogram was almost identical to that of size-matched controls (Figs 1b, 4f).

In untreated AH13r tumours, acidic metabolites were not readily cleared from the tissue, as indicated by the mean pH of 6.65 in both control groups. In addition, there was marked regional heterogeneity, with pH values ranging from 6.2 to 7.0 (Fig. 5a,b). CP administration had no immediate effect on intratumoral pH (day 1: mean pH 6.68; range 6.4–7.3) (Fig. 1c). However, on day 2 after treatment, the pH histograms underwent an alkaline shift: the frequency of pH readings < 6.5 was reduced to 2% as compared to 25% in control group II and the mean pH increased from 6.65 to 6.82 ($P < 0.001$; Figs 1c, 5c). The latter values correspond to a CP induced reduction of the concentration of hydrogen ions by ~ 33%. The alkaline shift persisted as the tumours continued to regress. At a tumour volume of ~ 1.0 cm^3 (day 4) the mean pH was 6.81 (range 6.5–7.1) ($P < 0.001$; Figs 1c, 5d). During early tumour regrowth, pH histograms returned to the values of size-matched controls, a process first apparent on day 8 (Fig. 5e). The kinetics of CP-induced changes in both pO_2 and pH are summarised in Fig. 1b and c.

DISCUSSION

In untreated AH13r tumours, pO_2 as well as pH values were significantly lower than in normal tissues [29], indicating an imbalance between the metabolic activity of the tumour cells and the vascular and interstitial transport of substrates and metabolites. This imbalance was volume-dependent: a 2-fold increase in tumour volume was accompanied by a 25% reduction of the mean pO_2. Following subcurative CP treatment, tumour oxygenation improved, a process apparent as early as 24 h after drug administration and persisting until the tumours escaped from growth delay. On day 2, the intratumoral pO_2 reached its maximum. At this time, all oxygenation values exceeded those of size-matched controls. It should be noted, however, that the rise of tumour pO_2—as expressed in terms of increasing frequencies of the higher classes of pO_2 values—did not involve all tumour areas to the same extent.

Tumour reoxygenation was paralleled by a shift of pH to more alkaline values. This increase in intratumoral pH occurred with similar kinetics as the changes in pO_2. Again, the maximum CP-mediated effect, i.e. an increase in pH by ~ 0.2 units, was observed between day 2 and day 4 after treatment. During tumour regrowth, and concomitant with increasing hypoxia, the pH returned to control values. These results indicate that the CP-induced reduction in the number of viable cells in AH13r tumours leads to a transient decrease in oxygen consumption as well as in lactate production, as reflected by the increase in intratumoral pH and pO_2. When displayed on a scale

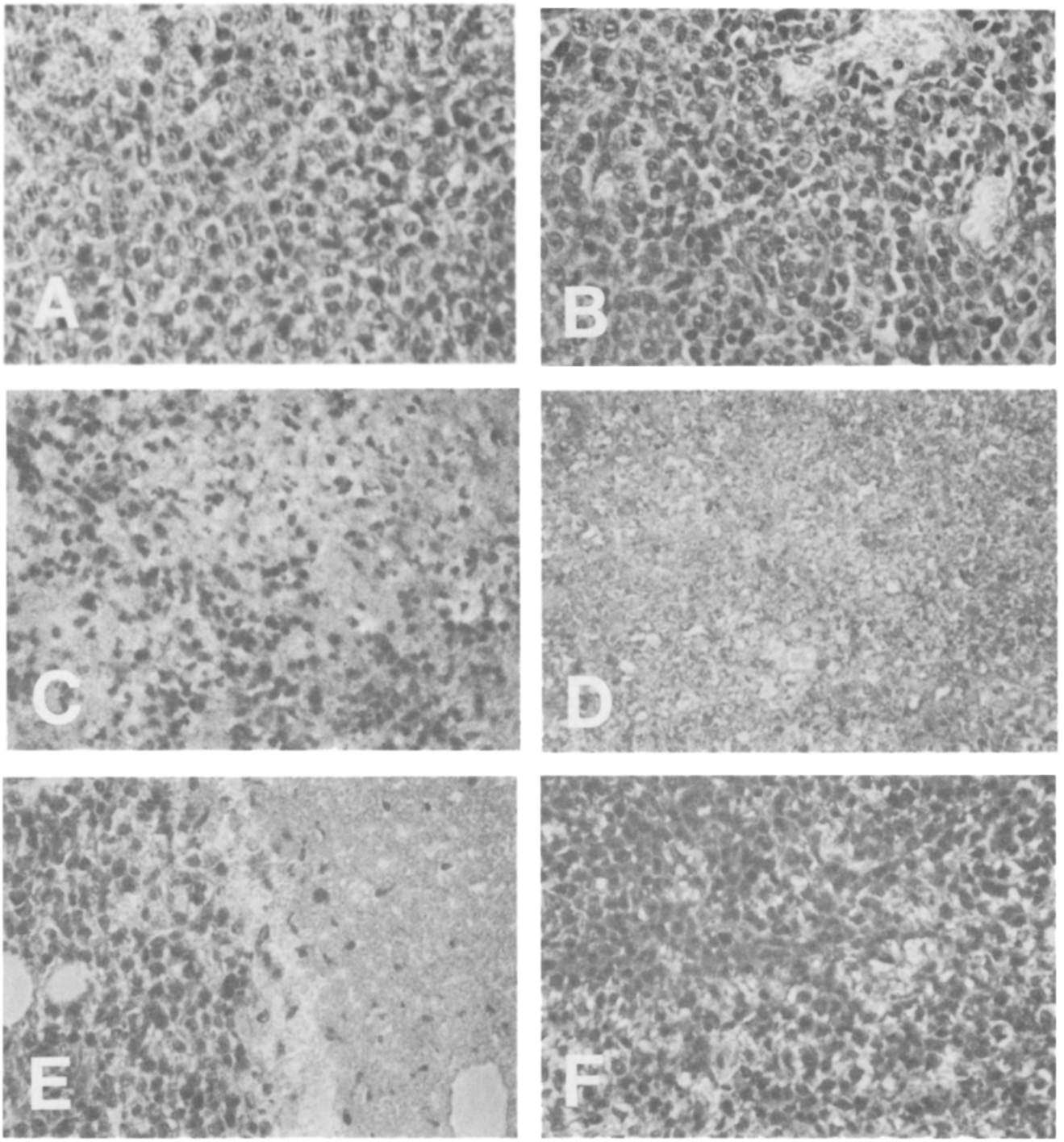

Fig. 2. Effect of CP treatment (40 mg/kg) on the histological appearance of AH13r tumours. Original magnification, ×100. (a) Control group I; (b) control group II; (c)–(f) days 2, 4, 8 and 12 after CP treatment, respectively.

encompassing the whole range of values measured in different tissues (Figs 3 and 4), the treatment-induced changes in pH and pO_2 appear relatively small. However, as discussed below, they may be sufficient to modulate the response of tumour cells to various treatment modalities.

To the best of our knowledge, this is the first study in which changes in pH and pO_2 following cytotoxic treatment have been measured *simultaneously* using *invasive* techniques. In most previous studies, treatment-induced changes in tumour metabolism have been quantitated indirectly, for example by *ex vivo* cell survival assays (pO_2) or non-invasively by ^{31}P-NMR spectroscopy (intracellular pH) [20, 21, 31, 32]. With regard to pO_2, and irrespective of quantitative differences between various types of tumours, our results extend previous reports describing a decrease in the fraction of radiobiologically hypoxic cells following radiation treatment to similar changes induced by chemotherapy [31–33]. The alkaline pH shift demonstrated in AH13r tumours following CP administration corroborates reports by other investigators using ^{31}P-NMR spectroscopy for pH measurements [20, 23, 34, 35]. In some cases, however, NMR studies have given conflicting results [20]. In evaluating these investigations, one has, however, to consider that pH

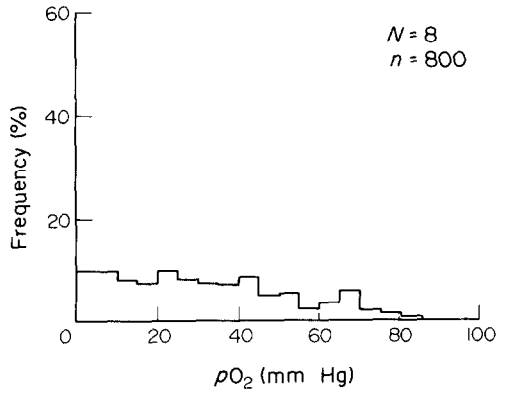

Fig. 3. Oxygenation of rat thigh muscle. *N*, Number of muscles; *n*, number of pO_2 readings.

measurements performed with (semi-)microelectrodes cannot be compared directly with NMR studies. Whereas the former are generally accepted to indicate the H^+ ion activity in the extracellular space and in cell debris created by the electrode track, the latter are a measure predominantly of intracellular pH [22–25, 34]. On the other hand, although mammalian cells

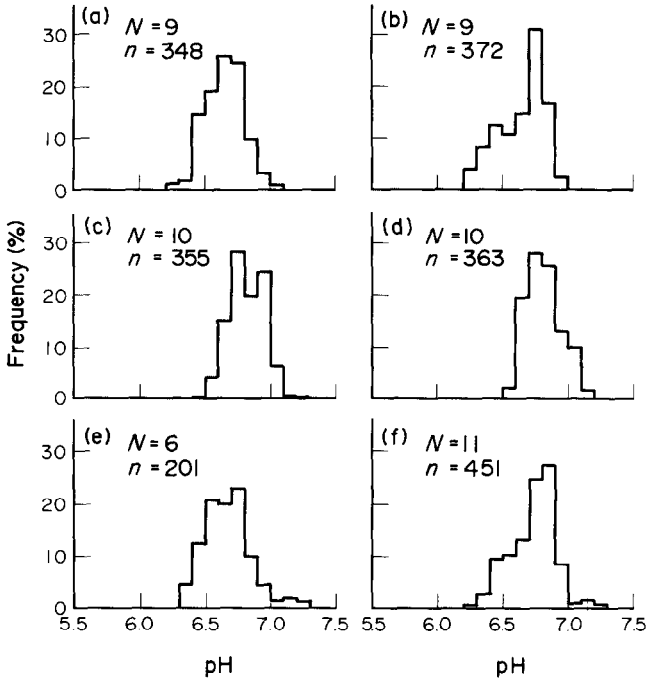

Fig. 5. Physiological response of AH13r tumours to CP treatment. II: pH. Designations as in Fig. 4.

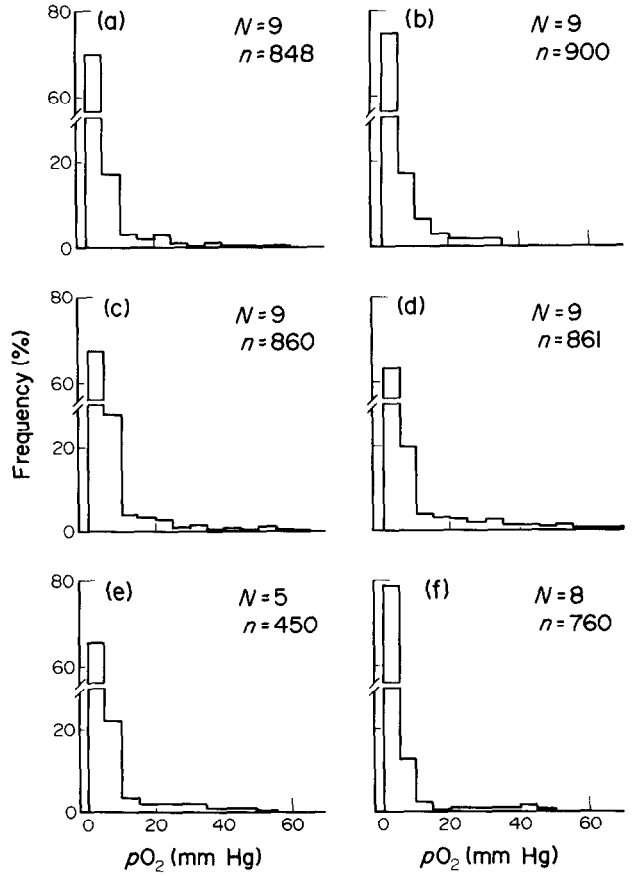

Fig. 4. Physiological response of AH13r tumours to CP treatment. I: oxygenation. pO_2 histograms were measured on day 3 (a), immediately prior to treatment (day 0) (b), and on days 2 (d), 4 (c), 8 (e) and 12 (f) following CP (40 mg/kg) administration. Note that histograms a and d, as well as histograms b and f, represent paired pre-treatment and post-treatment tumours of the same size. *N*, number of tumours; *n*, number of pO_2 readings.

are able to maintain a H^+ ion gradient across plasma membranes, marked alterations of extracellular pH can be accompanied by parallel—although not equivalent—changes of intracellular pH [16].

Various attempts to optimise chemotherapy aim at circumvention or reversal of drug resistance. These include investigations into the relative impact of various mechanisms determining cellular drug sensitivity such as transmembrane drug transport, drug metabolism and DNA repair. A complementary approach could be the design of treatment protocols which take advantage of analyses of microenvironmental determinants of drug activity, with regard to both the selection and the scheduling of therapeutic agents. This concept is based on observations indicating that cytotoxic effects of anti-cancer agents can, in certain settings, be more dependent on environmental factors than on the intrinsic drug sensitivity of the target cells [1–5]. For the clinical treatment of malignant tumours, agents are available which exhibit their maximum activity against either hypoxic or well-oxygenated cells and at either acidic or alkaline pH. Therefore, therapeutic effectiveness might be improved by treatment protocols taking into account (i) the pretreatment status and (ii) therapy-induced changes of microenvironmental determinants of target cell drug sensitivity.

In untreated AH13r tumours, ~ 75% of the cells were exposed to local oxygen tensions < 5 mmHg, a value close to the limit of hypoxia in radiobiological terms. On day 2 after CP treatment, this fraction was reduced to ~ 50%. The magnitude of these changes appears sufficient to facilitate the proliferative recovery of quiescent hypoxic cells after treatment and to improve the effectiveness of radiation therapy by optimal timing [12, 36]. It is a more difficult question whether these changes could also modulate the effect of consecutive courses of drug therapy—apart from an increment in chemoresponsiveness due to cell recruitment [37].

Most studies on the dependence of drug cytotoxicity on oxygen partial pressure have been performed on malignant cells

exposed to an atmosphere of either N_2/CO_2 or air/CO_2 (for review, see [17, 38]). Although marked differences in drug effects have been described at these extremes, there are only a few data on the effects of small increments in pO_2, as demonstrated in this study in post-treatment AH13r tumours. From the work of Yamauchi *et al.* [39] it can be estimated that the cytotoxicity of bleomycin and etoposide, as measured by the relative drug concentrations required to achieve equivalent cell killing, increased by about 50–100% when the oxygen concentration was raised from 0 to 2.5%. Conversely, the potency of mitomycin C was reduced by about 25% when pO_2 was raised from 5 to 7.5 mmHg [38]. Similar results have been reported for misonidazole [40, 41].

There are two classes of anti-cancer drugs whose cytotoxicity is particularly, albeit inversely, sensitive to changes in pH: anthracyclines, including the anthracenedione mitoxantrone, and alkylating agents [42–44]. Thus, when the microenvironmental pH of cultured rat mammary carcinoma (M1R) cells was raised from 6.5 to 6.8—a pH shift close to the post-treatment changes observed in AH13r tumours—the cytotoxicity of doxorubicin (as measured by the survival of colony forming cells) increased by 1 log. The latter value corresponds to a 2-fold increase in doxorubicin potency, as expressed in terms of the concentrations required to achieve equivalent cytoreduction. Similar results have been obtained with daunomycin, epirubicin, and, in the pH range between 6.8 and 7.4, with mitoxantrone [42, 43]. Conversely, the activity of alkylating agents is reduced at alkaline pH as compared with acidic pH. However, the magnitude of the pH-dependent changes in cytotoxicity is less pronounced than that observed with anthracyclines. For example, in order to decrease by 50% the potency of mafosfamide, a precursor of activated cyclophosphamide, the pH of cultured M1R cells had to be raised by 0.6 units from 6.8 to 7.4 [16].

In the AH13r rat tumours analysed in the present study, the changes in pO_2 as well as in pH occurring subsequent to an estimated 1.8-log cell kill are sufficient to allow for an increased drug sensitivity equivalent to a 25–100% dose escalation. In clinical trials, increases in dose intensity (i.e. the amount of drug delivered per unit time) of this magnitude have been associated with substantial improvements in disease-free survival (for review, see [45]). It is, of course, difficult to extrapolate, in quantitative terms, from experimental studies in transplantable rodent tumours to the more complex situation in clinical cancer therapy. Nonetheless, the present data support the view that the therapeutic index of anti-cancer agents in appropriately designed protocols for combination chemotherapy or multimodality treatment can be improved by taking into account treatment-induced changes in the internal milieu of tumours. Specifically, it may be concluded that treatment should be initiated by administration of alkylating drugs or hypoxic cell cytotoxins activated by reductive metabolism [7, 46] and, only after an interval of several days, followed by the application of, e.g. anthracyclines and/or irradiation.

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Acknowledgements—We are indebted to Professor C. Weiss for critical discussions and to G. Jost, K. Heise and M. Zaczek for expert technical assistance. Supported by grant W 15/88 Ra 3 from the Dr Mildred Scheel Stiftung für Krebsforschung.

Eur J Cancer, Vol. 29A, No. 1, pp. 122–123, 1993.
Printed in Great Britain

0964–1947/93 $5.00 + 0.00

Survival Trends for Neuroblastoma Patients in Finland: Negative Reflections on Screening

Risto Sankila and Matti Hakama

Based on 257 neuroblastoma patients in the age group 0–14 years and reported to the Finnish Cancer Registry, the 5-year cumulative survival rates have improved from 15% in the 1950s to 57% in 1980–1986. The potential benefit of screening for neuroblastoma was assessed on the basis of these nationwide survival trends. It is likely that any decrease in the overall neuroblastoma mortality due to screening would be small, because the survival rates of the Finnish neuroblastoma patients are already, even without screening, similar to those in Japan, which has a nationwide public health policy to screen for neuroblastoma.
Eur J Cancer, Vol. 29A, No. 1, pp. 122–123, 1993.

INTRODUCTION

NEUROBLASTOMA IS the third most common tumour among children after leukaemias and brain tumours [1]. Screening for neuroblastoma is technically possible by detecting the excess amounts of catecholamine metabolites in urine excreted by most of the tumours [2]. The purpose of this study is to estimate the survival rates of neuroblastoma patients in Finland without any screening. The rates and trends are available at the population-based, nationwide Finnish Cancer Registry which has been functioning since 1953. By comparing the Finnish rates to those reported in Japan, where a nationwide policy for screening for neuroblastoma has been established, we assess the likely benefit of introducing a screening programme for neuroblastoma.

PATIENTS AND METHODS

The population-based, nationwide Finnish Cancer Registry has been functioning since 1953. All hospitals, physicians and pathological laboratories are required to notify the Registry of all cancer cases that come to their attention. The Registry also receives information on all death certificates in which a cancer diagnosis is mentioned.

Between 1953 and 1986 altogether 259 histologically verified neuroblastomas and ganglioneuroblastomas in the age group of 0–14 years were reported to the Finnish Cancer Registry. 2 of

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Revised 23 Mar. 1992; accepted 28 Apr. 1992.