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Possible Coexistence of Two Independent Mechanisms Contributing to Anthracycline Resistance in Leukaemia P388 Cells

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Murine leukaemia P388 and L1210 cell sublines with varying degrees of resistance to the anthracycline daunomycin (DNM) have been used to monitor (i) intracellular accumulation of DNM, (ii) expression of the drug efflux pump P-glycoprotein (pgp) and (iii) cytoplasmic pH changes. Drug-resistant L1210/65 cells (65-fold resistance), overexpress pgp, and display decreased intracellular accumulation of DNM and identical intracellular pH as compared to the parental drug-sensitive L1210 cell line. On the other hand, moderately drug-resistant P388/20 cells (20-fold resistance), which also exhibit a decreased intracellular drug accumulation with respect to drug-sensitive P388/S cells, display only moderate pgp-encoding *mdr*1 gene transcription without detectable levels of pgp protein, and undergo cytoplasmic alkalinisation (up to ~0.2 pH units). A further increase in the level of drug resistance (P388/100 cells, 100-fold resistance), results in a more pronounced decrease in drug accumulation, significant pgp expression and slightly higher intracellular alkalinisation. Alterations in the degree of protonation of DNM have been shown previously to influence processes such as the rate of uptake and the intracellular accumulation of the drug. On this basis, we propose that the changes in intracellular pH, observed at low levels of drug resistance (P388/20 cells), could constitute an early cellular response aimed at decreasing the intracellular accumulation of ionisable anti-neoplastics. As the level of resistance increases (P388/100), the cells seem to require more efficient mechanisms of defense against the drug, such as that represented by the expression of pgp. Since there is no apparent correlation between the extent of the changes in intracellular pH and the level of pgp expression in DNM-resistant P388 cell sublines, it is suggested that these two cellular responses contributing to drug resistance could operate independently.
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INTRODUCTION

WHEN CONFRONTED with cytotoxic agents, tumour cells having the multidrug resistant (MDR) phenotype frequently exhibit reduced intracellular accumulation of the drugs with respect to the parental drug-sensitive cells [1]. An increased drug efflux has been used to explain this property of resistant cells, based on the overexpression of certain membrane glycoproteins, the P-glycoprotein (pgp) family, responsible for actively pumping the drugs out of the cells [2]. However, recent findings on neuroblastoma cells suggest that the overexpression of pgp does not modify the extent of drug accumulation [3, 4]. Also, there are a number of drug-resistant cell lines and tumours in which overexpression of pgp has not been

detected ([5] and references therein). This evidence suggests that cellular alterations, other than the overexpression of pgp, could also be involved in lowering the intracellular accumulation of drugs. In this context, some of the mechanisms which have been considered include increased intracellular membrane traffic [6], overexpression of vacuolar ATPases [7] and alteration of cytoplasmic pH (pH_i) [8–10]. With regard to the latter possibility, it should be noted that anthracyclines, such as daunomycin (DNM), are potent antitumour agents [11], bearing an ionisable amino group at the sugar moiety daunosamine, with a pK_a between 7.6 and 8.2 [12]. Thus, at physiological pH, the anthracyclines exist in equilibrium between neutral and ionised species. The cells are more permeable to the neutral than to the ionised forms of the drugs, while the latter have a higher affinity for several cellular targets, including nucleic acids [13], phospholipids [14], nucleotides [15] and tubulin [16]. Therefore, the predominance of either one of these forms at equilibrium should be relevant in determining retention of the drugs in intracellular acidic compartments, cellular drug permeability and drug cytotoxicity [6, 17].

In this paper, we report on the possible co-existence of two mechanisms, that is an increase in pH_i and pgp expression, accompanying the acquisition of resistance to DNM in leukaemia P388 cells. The relative contribution of either one of these mechanisms to drug resistance in the P388 cells seems to be associated with the level of resistance.

MATERIALS AND METHODS

Cell cultures

P388 and L1210 murine leukaemia cell lines were maintained in culture as described previously [18]. Stable DNM-resistant sublines were obtained by stepwise selection with increasing concentrations of DNM. Compared to the parental DNM-sensitive lines (P388/S and L1210/S), the DNM-resistant sublines P388/20, P388/100 and L1210/65 were ~20, 90–100 and 65-fold resistant to DNM, respectively, determined by *in vitro* proliferation assays described previously [19].

Intracellular pH determination

Loading of the cells with the fluorescence pH indicator 2′,7′-*bis*-(2-carboxyethyl)-5(6-carboxyfluorescein) (BCECF) (Molecular Probes, Eugene, Oregon, U.S.A.), was carried out as described previously [20, 21]. The buffer was 10 mmol/l Hepes (pH 7.2), containing 130 mmol/l NaCl, 5 mmol/l KCl, 1.8 mmol/l $CaCl_2$ and 1 mmol/l $MgCl_2$ (HBS solution). Loaded cells exhibited strong fluorescein emission when observed under a fluorescence microscope, and were more than 95% viable according to the Trypan blue exclusion test. Furthermore, permeabilisation of the cells with 0.005% digitonin resulted in negligible cellular fluorescence levels remaining, thus indicating the cytoplasmic location of the dye.

The measurements of pH_i were made in cellular suspensions, containing 2×10^5 BCECF-loaded cells in 2 ml of HBS solution, by using the "null point" method [22]. This method uses a mixture of a weak acid (butyric acid, pK 4.82) and a weak base (trimethylamine, pK 9.8), whose uncharged forms are able to cross the cell membrane at a similar rate, thus producing changes in pH_i. In practice, the experiment consists of finding which proportion of acid and base in the mixture produces no changes (null point) in the BCECF signal (i.e. in the pH_i). The pH_i value can then be calculated from the composition of the acid/base mixture [22]. In some instances, the values of pH_i determined by the null point method were compared to those obtained by two other methods which also use BCECF as the fluorescence pH indicator and the K^+/H^+ ionophore nigericin [23, 24]. The fluorescence intensity determinations were made in an SLM-8000 spectrofluorimeter equipped with a thermostated holder and magnetic stirrer, at 20°C. Excitation wavelengths were 500 and 440 nm, while emission wavelength was 530 nm. Contribution of scattered light was always negligible (<1%).

Drug accumulation studies

These experiments were carried out as described previously [25]. Briefly, cell suspensions (10^6 cells/ml) in HBS were incubated at 20°C in the presence of 3 μmol/l of either DNM or its aglycon form, daunomycinone. Daunomycinone was obtained by acid hydrolysis of DNM [26]. At predetermined time points, aliquots from the incubation mixtures were subjected to flow cytometry in an Epics Profile I instrument equipped with a 0.75 W argon laser set at 488 nm, interfaced with an IBM data acquisition and analysis system. Data analysis included the quantitation of (i) the number of cells having a significant content of anthracycline (i.e. a level of drug fluorescence which excludes cell autofluorescence, drug association to cell debris, etc), and (ii) the mean fluorescence intensity (mean fluorescence channel in the histograms) which, for a given cell population, is proportional to the concentration of drug associated with each cell. A total of 10^5 cells were measured during each sample analysis. Cell viability at the end of the experiments was similar to that of the starting samples (>95% viable cells).

Pgp determination

Presence of *mdr* mRNA was determined by northern blot analysis. Analysis of pgp protein was based on immunoreactivity against the C219 mouse IgG_{2a} monoclonal antibody (PGlycoCHECK, Centocor Inc., Malvern, Pennsylvania, U.S.A.).

Northern blot analysis. Total cellular RNA from DNM-sensitive and DNM-resistant P388 cells was isolated by cell lysis in guanidinium isothiocyanate and phenol extraction [27], electrophoresed on 1% agarose/2.2 mol/l formaldehyde gels and transferred onto Nylon membranes [28]. Blots were hybridised with the pCPH1 probe (generously provided by Dr V. Ling, Ontario Cancer Institute, Toronto, Canada). Hybond-N membranes containing fractionated RNAs were prehybridised in 50% formamide, 5 × Denhart solution, 0.01% SDS, 5 × SSC (1 × SSC is 0.015 mol/l sodium citrate, pH 7.0, 0.15 mol/l NaCl) and 100 μg/ml salmon esperma DNA for 4 h at 42°C. Filters were then hybridised as described previously [28] for 16–20 h at 42°C in the same buffer as DNA probes, labelled with [α-^{32}P]-dCTP by random priming [29]. For the RNA blots, final posthybridisation washes were at 2 × SSC plus 0.01% SDS at 42°C. Blots were visualised by autoradiography and the relative intensities of the bands were quantified using a Bio-Rad model 620 video densitometer. The β actin probe gene (kindly provided by Dr F. Ruiz Cabello, Immunology and Clinical Trial Service, Hospital Virgen de las Nieves, Granada, Spain) was employed for normalisation, as described previously [30].

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Flow cytometry. Determination of the pgp protein content was performed using the fluorescein-labelled monoclonal antibody (mAb) C219 (C219 fluorescein isothiocyanate) [31]. A fluorescein-labelled, non-reactive IgG_{2a} monoclonal antibody was also used to evaluate background fluorescence due to non-specific antibody binding. Normal mouse lymphocytes were employed as negative control for overexpression of pgp. The quantitation of the levels of pgp in the different cell lines was estimated by measuring (i) the percentage of fluorescent-positive cells when the negative control was set to be 5% positive [32, 33], and (ii) the mean fluorescence intensity, which is proportional to the amount of bound antibody per cell.

Western immunoblots. Microsomal membrane proteins from each cell line were obtained as described previously [18], except that the last sucrose density gradient step was omitted. Protein determinations were carried out by the method of Lowry *et al.* [34]. The microsomal proteins were fractionated by SDS–PAGE [35] and transferred electrophoretically to nitrocellulose [36]. Non-specific binding was prevented with a solution of 2 mg/ml bovine serum albumin (BSA) in Dulbecco's PBS buffer, containing 0.1% Tween-20 (blocking buffer), for 18 h at 5°C. The transfers were incubated with the mAb C219 (0.2 μg/ml) in the same buffer at room temperature for 18 h. The blots were finally submitted to four 5-min washes with blocking buffer without BSA, and incubated with goat anti-mouse IgG conjugated to alkaline phosphatase (Boehringer Manheim Biochemicals, Germany) in blocking buffer at room temperature for 3 h. After four additional washes (5 min each) with 100 mmol/l Tris–HCl (pH 8.8), 100 mmol/l NaCl, 5 mmol/l $MgCl_2$ (buffer A), alkaline phosphatase activity was detected by incubating the blots with a solution containing 3.75% of 4-nitro blue tetrazolium chloride, 15% of 5-bromo-4-chloro-3-indolilphosphate in buffer A. The developing reaction was stopped by washing the transfer with distilled water.

RESULTS

Table 1 summarises the cytoplasmic pH values, corresponding to DNM-sensitive and DNM-resistant P388 and L1210 cells, obtained with the fluorescent pH indicator BCECF, which shows a linear response in the 6.4–7.6 pH range [37]. The null point method [22] to determine pH_i was preferred because (i) the leakage of BCECF was ~1% per hour versus 25–30% per hour found for the other two methods [23, 24] and, (ii) while the latter were time-consuming assays (20–25 min for each pH determination), the null point method needed less than 10 min per sample. Regardless of the method used, drug-resistant P388 cells consistently exhibited higher pH_i than the drug-sensitive P388 cells. The pH_i increases with the level of resistance: the most drug-resistant cell line P388/100 showed a higher pH_i than moderately resistant P388/20 cells, which in turn, displayed a higher pH_i than P388/S cells. In contrast, no differences in pH_i were observed between DNM-sensitive and DNM-resistant L1210 cells, both of which exhibit pH_i values similar to that presented by the P388/100 cells.

Table 1. Intracellular pH values of DNM-sensitive (P388/S) and DNM-resistant cell sublines (P388/20, P388/100), estimated with the fluorescent pH indicator BCECF

Cell line	pH*	pH*	pH*
P388/S	6.89 ± 0.02	6.81 ± 0.05	6.79 ± 0.02
P338/20†	7.11 ± 0.01	7.10 ± 0.04	7.08 ± 0.02
P388/100†	7.20 ± 0.02	N.D.	N.D.
L1210/S	7.29 ± 0.02	N.D.	N.D.
L1210/65	7.26 ± 0.01	N.D.	N.D.

Values are given as pH units ± S.D. (n=4). N.D., not determined. *pH_i values determined by the null point method and by those described in [23] and [24], respectively. † Significant difference from control ($P<0.05$, Student's t-test).

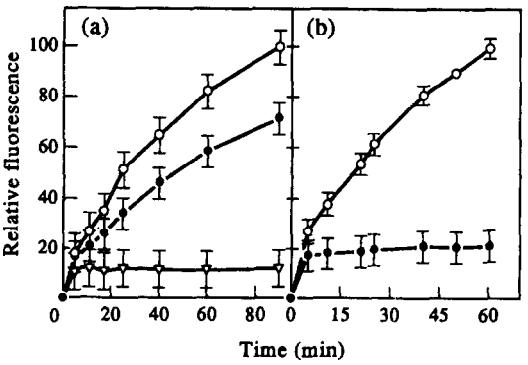

Fig. 1. Time-dependent uptake of DNM by P388 cells as estimated by flow cytometry. Cells were incubated with 3 μmol/l of DNM in HBS solution at pH 7.2 and 20°C. The y axis indicates the normalised percentage of drug fluorescence associated with each cell population (mean fluorescence channel in the histograms). One hundred per cent refers to the level of fluorescence exhibited by drug-sensitive cells. Panel a: (○) P388/S cells; (●) P338/20 cells; (▽) P388/100 cells. Panel b: (○) L1210/S cells; (●) L1210/65 cells. Bars indicate ± S.D.

In order to evaluate the intracellular accumulation of DNM in the P388 and L1210 cells, we measured the total drug uptake by flow cytometry under experimental conditions identical to those used previously to determine intracellular pH. The uptake of DNM at preselected times is represented in Fig. 1, in terms of the changes in the fluorescence intensity exhibited by the different cell populations (mean fluorescence channel in the flow cytometer). As expected from other reports [1, 25, 32, 33, 38], accumulation of DNM in drug-resistant cells was consistently lower than that in drug-sensitive cells.

To test the possible influence of the pH_i changes observed in the P388 cells (Table 1) on the uptake of the ionisable DNM, we determined the intracellular accumulation of the aglycon daunomycinone, which lacks the ionisable sugar amino group of DNM. As illustrated in Fig. 2, no differences in the intracellular

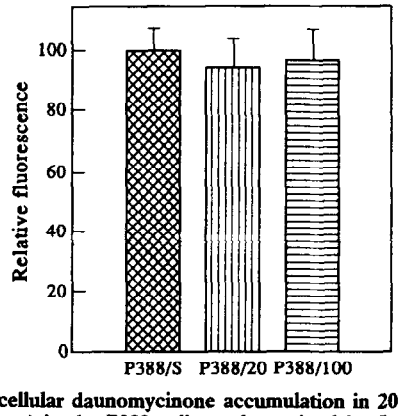

Fig. 2. Intracellular daunomycinone accumulation in 20 min (steady-state conditions) in the P388 cells as determined by flow cytometry. Incubation conditions were as in Fig. 1. The y axis indicates the percentage of drug fluorescence as defined in Fig. 1. Bars indicate ± S.D.

steady-state level of daunomycinone (which occurs in ~20 min), between drug-sensitive and drug-resistant P388 cells were detected.

Because differences in drug accumulation between drug-sensitive and drug-resistant cells are frequently explained as the result of an enhanced, active drug efflux in the latter via pgp [2, 39–40], we determined pgp expression in the P388 and L1210 cell lines. Presence of pgp protein was determined by examining the ability of the cells to bind the MAb C219, which recognises a cytoplasmic epitope common to all the pgp family members [41] and enables the detection of the pgp protein, even in cell lines of low drug-resistance [42]. Figure 3 shows the results obtained when flow cytometry was used to monitor MAb labelling of permeabilised P388 and L1210 cells. It was observed that the P388/S and the P388/20 cells (Fig. 3b and c, respectively), exhibit similar low levels of fluorescence labelling by the antibody, regardless of whether the histograms were analysed in terms of the number of fluorescent-positive cells or in terms of the mean fluorescence intensity associated with each cell population. Furthermore, reactivity against MAb C219 exhibited by these cells was almost identical to that observed in normal mouse lymphocytes used as a negative control (Fig. 3a). In contrast, an increase in antibody labelling was observed in P388/100 cells (Fig. 3d) suggesting that, as reported by others, pgp in P388 cells may be expressed in highly resistant cell lines [42]. In the case of L1210 cells, expression of pgp is readily detectable in DNM-resistant L1210/65 cells (Fig. 3e), while DNM-sensitive L1210/S cells display similar values to those found in P388/S cells or in normal mouse lymphocytes (data not shown). Compared to similar flow cytometry analysis reported in other tumour cell lines to quantitate pgp [32, 33], our P388/100 and L1210/65 cells should be considered as moderately overexpressing pgp (values between 10 and 20% of fluorescent-positive cells have been considered as intermediate for the overexpression of the antigen). The flow cytometry results were confirmed by western immunoblots of protein samples from microsomal membrane preparations, obtained from each of the cell types using the MAb C219 which has been employed specifically to detect pgp on immunoblots [43]. As shown in Fig. 4, the parental drug-sensitive L1210/S and P388/S cells (lanes 2 and 5, respectively), as well as the moderately resistant P388/20 cells (lane 4), do not display protein bands with immunoreactivity against the antibody. However, protein samples from the L1210/65 (lane 1) and P388/100 cells (lane 3), show a positive immunolabelled protein band, with an apparent molecular mass in the range of that reported for pgp (170–180 kDa), thus confirming further the presence of the protein in these latter samples.

Because of the lack of pgp detection in the P388/20 cells, we also measured *mdr*1 gene transcription in all the P388 cell sublines, because of the higher sensitivity of these assays compared to those used in the detection of pgp with the C219 antibody. As shown in Fig. 5a, northern blot analysis from total RNA reveals a major transcript of approximately 4.7 Kb, similar to the size of the RNA reported for the mammalian *mdr*1 gene [44, 45], that was recognised by the pCPH1-specific probe. The hybridised signals on P388/20 and P388/100 cells were 14- and 50-fold, respectively, to that observed in P388/S cells. A probe encoding the β-actin gene was employed for normalisation of the amount of RNA (Fig. 5b).

DISCUSSION

The decreased capacity of MDR cells to accumulate antitumour drugs, as compared to that observed in the parental drug-sensitive cells, is explained by either changes in the affinity of the drugs for intracellular targets or alterations in the drug transport. While few attempts have been made to provide experimental evidence to support the former hypothesis [1, 46], much more attention has been devoted to the latter, partly because of the demonstrated overexpression of energy-dependent drug efflux pumps (the pgp family), which are commonly found in drug-resistant tumour cell lines [2]. Nevertheless, there is increasing evidence to suggest that overexpression of pgp, increased drug efflux and decreased drug accumulation do not necessarily correlate [5], thus suggesting that additional mechanisms should also be involved in the development of drug resistance in tumour cells.

In this study, we have observed that DNM-resistant L1210

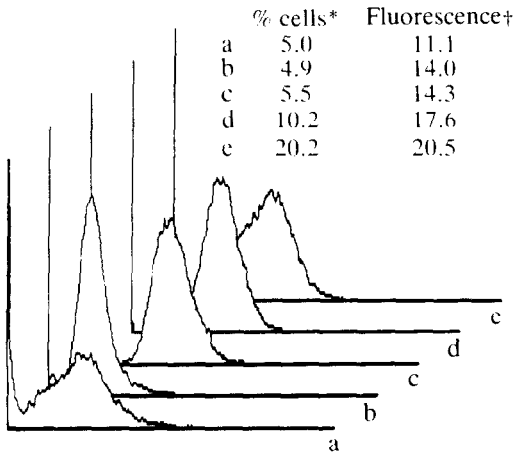

Fig. 3. Cellular FITC-labelled MAb C219 fluorescence in P388 and L1210 cells obtained by flow cytometry to determine the relative content of P-glycoprotein. On the abscissa, the relative fluorescence is recorded while the height of the histograms is proportional to the number of cells having a fluorescence corresponding to a particular channel value. (a) Normal mouse lymphocytes (negative control); (b) P388/S cells; (c) P388/20 cells; (d) P388/100 cells; (e) L1210/65 cells. Inset values include: *the percentage of fluorescent-positive cells and †the average fluorescence intensity (mean fluorescence channel), associated with each cell population (see Materials and Methods).

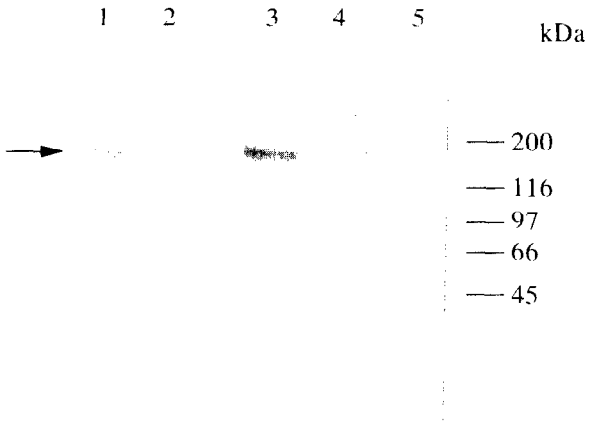

Fig. 4. Detection by western immunoblot of the binding of the MAb C219 to pgp present in microsomal membrane fractions obtained from P388 and L1210 cells. Aliquots containing 20 μg of protein from microsomal fractions from L1210/65 (lane 1), L1210/S (lane 2), P388/100 (lane 3), P388/20 (lane 4) and P388/S (lane 5), were resolved by SDS–PAGE and transferred to nitrocellulose. Antibody binding was detected using goat anti-mouse IgG conjugated to alkaline phosphatase. The arrow indicates the position of the pgp. Positions of molecular mass markers are shown on the right.

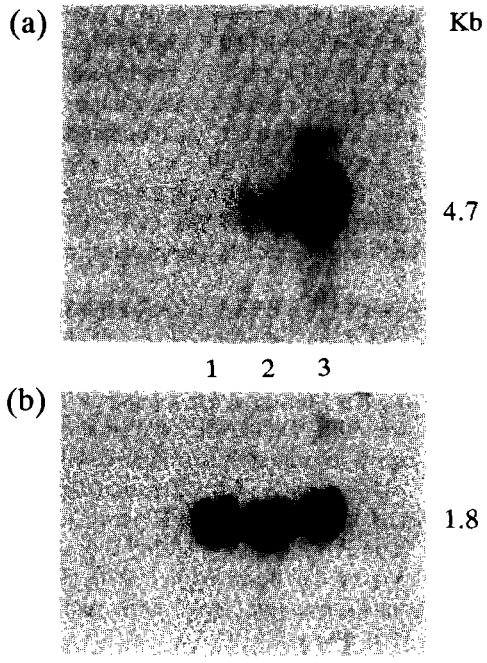

Fig. 5. Northern blot analysis of total RNA from the P388 cells. (a) Total cellular RNA (20 μg) isolated from P388/S (lane 1), P388/20 (lane 2) and P388/100 cells (lane 3) was resolved by electrophoresis transferred to nylon membranes and sequentially hybridised with ^{32}P-labelled pCPH1 probe. (b) The blot was stripped and reproved with ^{32}P-labelled β actin probe. Transcript sizes were estimated relative to the migration of 18 and 28S rRNA.

and P388 cells behave similarly in terms of drug accumulation (resistant sublines accumulate less drug than sensitive cells) and pgp expression, but they differ in that while L1210/65 cells do not undergo changes in pH_i, with respect to the parental drug-sensitive cell line, DNM-resistant P388 cells exhibit a significant cytoplasmic alkalinisation.

While the functional role of pgp has been extensively documented as a very efficient mechanism to reduce intracellular drug accumulation [2], there is also evidence to suggest that alterations in pH_i could be involved in changing the extent of retention of ionisable anticancer drugs by the cells [8–10]. In relation to this, Skovsgaard and Nissen [12] indicated that an increase in the pH of the media, with respect to the cell interior, favoured the inward transport of DNM, leading to an increase in the intracellular steady-state level of the drug, a finding that has also been observed in artificial lipid vesicles subjected to a transmembrane pH gradient (interior acidic) [47]. This is similar to the situation described in our drug accumulation experiments, using the P388 cells which are in fact made in the presence of a pH gradient. This gradient is approximately 0.3 pH units in the case of P388/S cells, decreasing to 0.1 pH units in the P388/20 cells and practically disappearing in the P388/100 cells. Because the ionised species of anthracylines have a higher affinity for cellular targets [48], it is expected that the extent of intracellular binding of anthracyclines is greater in drug-sensitive than in drug-resistant P388 cells, on the basis of simple laws of chemical equilibrium. These expectations are consistent with a recent theoretical model based on Ehrlich ascites tumour cells, which predicts that an increase in the cytoplasmic pH from 6.95 to 7.40 would be sufficient to reduce by 35% the steady-state level of accumulation of daunomycin [49]. Furthermore, anthracyclines have been found to accumulate in acidic intracellular organelles [6, 17], an event that would also be favoured by the occurrence of a pH gradient between the cytoplasm and acidic compartments. Because DNM-resistant P388 cells have a higher cytoplasmic pH than DNM-sensitive P388 cells, drug trapping into intracellular acidic compartments should also be facilitated in the former. In addition, it has been reported that the acid endosomal compartment in drug-resistant P388 cells is significantly increased compared to that in drug-sensitive P388 cells [6]. Drug trapping in the endosomal–lysosomal complex and subsequent exocytosis of the drug to the external medium has been proposed as a mechanism to reduce intracellular drug accumulation in MDR cells [50]. Based on the above observations, we propose that the increase in pH_i observed in the drug-resistant P388 cells implies at least two possible, complementary effects to partly account for the lowering of intracellular drug accumulation: first, a decrease in the overall affinity of the drug for binding to cellular sites (due to a higher relative abundance of neutral versus ionised species of DNM) and second, an increased accumulation of DNM into acidic compartments, which could be followed by drug exocytosis. The relevance of the ionisation state of the drug to its intracellular accumulation in the P388 cells seems to be further supported by the observations on uptake of the aglycon daunomycinone, which has no ionisable groups at the pH used in the studies, and resulted in indistinguishable intracellular drug levels between drug-sensitive and drug-resistant cells.

The question remains as to whether or not the increases in pH_i and pgp expression represent independent cellular responses contributing to drug resistance. A previous study in a human tumour lung cell system [9] reported a correlation between pgp expression, increase in pH_i and the level of resistance, and in fact, these authors proposed the enhanced pgp activity as responsible for the progressive cytoplasmic alkalinisation observed in these cells. Furthermore, it has been reported that pgp may act as a chloride channel [51], thus providing a possible basis for this protein to exert an effect on the cytoplasmic pH in drug-resistant cells. However, our observations do not support the generalisation of such conclusions because first, there is no apparent correlation between the extent of the changes in pH_i and the level of pgp expression in the P388 cells. If this was so, a pH_i in the P388/100 cells higher than that measured should be expected. Second, the results obtained in the DNM-resistant L1210 cells indicate that the pgp expression does not necessarily imply cytoplasmic alkalinisation of the cells. Thus, we propose that the increase in pH_i and the expression of pgp could operate as independent mechanisms of drug resistance in the P388 cells. As indicated in the Introduction, drug resistance in different cell lines and tumours cannot always be explained solely on the basis of pgp overexpression. With regard to this, the increase in pH_i seen at low levels of resistance in the P388 cells prior to noticeable expression of pgp, could constitute a possible alternative for these cells and perhaps to other tumours to defend against ionisable drugs. Currently, however, there is only limited information on pH_i changes in other cell lines, and on its correlation with pgp expression and drug resistance. Therefore, it is difficult to predict how often a similar strategy is used by tumour cells in response to drug exposure.

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Side-effects of Screening

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There has been a 42% increase in the number of mammograms performed outside the national screening programme (operating in Camberwell, southeast London) which was not anticipated in the Forrest Report, a document to the Health Ministers of the U.K. by a working group chaired by Sir Patrick Forrest [1]. The report compiles recommendations on breast screening, using mammography and breast self-examination, to reduce the mortality in women aged 50–64 years [1]. This 42% increase is attributable mainly to referrals from menopause clinics and general practitioners of patients mainly in the screening age group. When we looked at referrals from general practitioners, suspicious mammographic findings were reported in 20% of patients referred with a breast lump, in contrast to only 4% of patients referred with breast pain or nodularity. Better education of both the public and general practitioners, concerning the signs and symptoms of breast cancer, may reduce demands to perform mammographies outside the current national screening programme.
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INTRODUCTION

IN AN attempt to reduce the mortality from breast cancer, routine mammographic screening in accordance with recommendations laid down in the Forrest Report [1], a document to the Health Ministers of the U.K. by a working group chaired by Sir Patrick Forrest, has been in operation in the Camberwell district of southeast London for approximately 18 months.

It has been our impression that there has been a substantial increase in the number of referrals in symptomatic women for mammography, creating an extra work load which was not originally anticipated in the Forrest Report [1].

In an attempt to validate our beliefs, we analysed the sources, the reasons and the outcomes of all referrals for mammography outside the screening programme.

MATERIALS AND METHODS

The hospital records of all requests for mammography outside the current screening programme (which is centred in Camberwell for the southeast district of London under the provision of the Forrest Report) were examined. It is stressed that these referrals were to the mammography unit and were separate from the current screening programme. The age of the patient, reason for referral and mammogram report were recorded in the subgroup referred from general practitioners (GP). Correlation between reasons for referral and patient complaints were obtained from each patient. If there was more than one reason the main problem was used in data analysis. The screening programme commenced in February 1988 and, at the time of investigation, had been in operation for approximately 18 months. Referrals during this time and for a similar period of time immediately before screening were recorded.

During the period of study, there were no changes in referral criteria for mammography. The referrals were from the same group of GPs before and during screening and, therefore, any increase was not a reflection of an increased population at risk from a larger catchment area. Referrals at the time of analysis were not affected by the present White Paper reforms within the National Health Service. Open access was available to both GPs and women attending the menopause clinic.

RESULTS

Between February 1986 and the end of July 1987 (before the start of the current screening programme) 1933 women were referred for mammography. The number of referrals after screening had commenced, during an identical period of time from February 1988 until 31 July 1989, was 2744 (an increase of 42%). The number of referrals from surgical outpatients and from the radiotherapy clinic remained approximately the same, however, substantial increases in referrals from menopause clinics and GPs were noted (Fig. 1).

Of the 614 referrals from GPs, the reason for referral was clear in 173 patients prior to screening and in 347 patients after screening had commenced. The main reasons for referral are listed in Table 1. They were reported as normal, or as having benign or suspicious changes present. The results of these mammograms in patients referred from GPs with a lump, breast pain or nodular breasts, before and after the onset of screening are shown in Fig. 2a–c. Over 40% of mammograms in women with a breast lump were reported as normal before and during screening, in contrast to women referred with breast pain or nodularity in which at least 80% of X-rays were reported as normal.

In contrast, between 15 and 23% of mammograms were

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