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Relations between the penetration, binding and average concentration of cytostatic drugs in human tumour spheroids*

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Summary. A penetration assay based on freeze-drying and vapour fixation was applied to show the spatial distribution of non-bound and bound cytostatic drugs in cellular spheroids. Several studies have proposed that peripheral binding of drugs correlates with limited penetration. We showed that granular accumulation, mainly at the peripheral part of spheroids, might occur in parallel with good penetration. For example, this was the case in human glioma spheroids after incubation with Adriamycin for 15–30 min. Following treatment with actinomycin D, colon carcinoma spheroids exhibited rather good penetration but also showed granular accumulation mainly in their peripheral regions. Ara-C accumulated largely and homogeneously in the peripheral regions of colon carcinoma spheroids and this severely delayed penetration. It took about 1 h for ara-C in the central regions of the spheroids to reach the same concentration as in the culture medium. In contrast, ara-C easily penetrated glioma spheroids without accumulating noticeably at the periphery. Retention tests involving washing and further incubation in drug-free culture medium revealed that the areas demonstrating extensive accumulation most often retained the drug, indicating binding, whereas the concentration of drug in other areas decreased. The oil-centrifugation method, which was used for rapid separation of the spheroids from the drug-containing medium, showed that the average concentration of daunomycin in the spheroids exceeded that in the culture medium as early as after 15 min, by which time only limited penetration had occurred. We found that good penetration of ara-C correlated with a low average concentration in glioma spheroids, whereas limited penetration correlated with a high average concentration in colon carcinoma spheroids. The latter finding was attributable to the high accumulation of drug at the spheroid periphery. Thus, there was an inverse relationship between penetration and binding and between penetration and average drug concentration. It seemed that binding delayed or prevented penetration, whereas little, if any binding resulted in better penetration. Granular binding such as that observed Adriamycin and actinomycin D gave intermediatley good penetration.

Introduction

Several studies have shown that some cytostatic drugs, such as anthracyclines, mainly bind and exert their toxic action in the peripheral regions of tumour nodules or in the outer cell layers of tumour spheroids [10, 12, 21, 31]. The relationship between penetration and binding is not known in detail. It has been proposed that the slow delivery of anthracyclines to the inner cells in spheroids is attributable to rapid binding in the outer layers [12]. In most studies in which limited penetration has been reported, the main difficulty has been that penetration per se was not studied. It is difficult to judge whether peripheral binding in a tumour nodule or tumour spheroid is attributable to limited penetration or whether the penetration is rather good but the binding occurs mainly in the peripheral regions. Conventional histology involving liquid fixation, dehydration and extensive handling of the sections washes away most substances that are not strongly bound to the cells or to the extracellular matrix. In such cases, only bound substances are left and peripheral binding might be interpreted as limited penetration. A similar situation, in which only bound drug remains for analysis, is encountered when tumour spheroids are enzymatically separated and the single cells are analysed, for example, using flow cytometry. A clear difference between binding and penetra-

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Table 1. Molecular weights and specific activity of the applied cytostatic drugs and the radioactivity and drug concentrations used in the penetration and liquid scintillation procedures

Drug	Mol. wt. Da	RADIOACTIVITY		
		Sp. act. (GBq/mmol)	Penetration (MBq/ml)	Liquid scintillation (MBq/ml)
[^{14}C]-Adriamycin	582	1.98	0.5 (145.0)[a]	0.02 (5.8)
[^{14}C]-Adriamycin	582	1.98	–	0.10 (29.0)
[^{14}C]-daunomycin	565	1.67	0.5 (169.0)	0.05 (16.9)
[^{3}H]-daunomycin	528	126	1.0 (4.2)	–
[^{3}H]-actinomycin D	1255	481	1.0 (2.6)	0.10 (0.26)
[^{3}H]-ara-C	245	1110	1.0 (0.22)	0.10 (0.022)
[^{3}H]-ara-C	245	414	1.0 (0.59)	–

[a] Drug concentrations (in µg/ml) are shown in parentheses

tion has recently been found for monoclonal anti-carcinoembryonic antigen (CEA) antibodies in colon carcinoma spheroids. The antibodies bound mainly in the outer regions of the spheroids, although the penetration was good [6]. A method based on freeze-drying and vapour fixation for direct analysis of penetration was used in that study, and the same method was applied in the present study.

The therapeutic response of tumour spheroids to cytostatic drugs has varied considerably in previous studies. It has been reported that Adriamycin, methotrexate, vincristine and vinblastine are taken up mainly in the peripheral cell layers of spheroids [9, 10, 12, 15, 22, 24, 26, 27, 32, 36], whereas 5-fluorouracil and cisplatin penetrate well [9, 10, 16, 20, 23, 26, 27]. It has also been found that spheroids are often more drug-resistant than the corresponding monolayers. One possible and often discussed explanation is that the drugs have difficulty penetrating the spheroids and that this contributes to the observed resistance [3, 12, 21, 31]. The relationships between the penetration, binding and average concentration of the four cytostatic drugs Adriamycin, daunomycin, actinomycin D and ara-C were analysed in colon carcinoma HT-29 and glioma U-118 MG spheroids of human origin. These drugs are well known and their mechanisms of action have been well characterised. The two anthracyclines (Adriamycin and daunomycin) and actinomycin D are taken up in cells by processes that do not require specific carriers or metabolic energy [34]; they intercalate DNA and are cytotoxic throughout the cell cycle. Ara-C, an analogue of deoxycytidine, is taken up in cells by nucleoside-specific membrane transporters [30] and acts as a "false base" to inhibit DNA replication [7].

Multicellular spheroids were used as models of tumour nodules to impose possible penetration barriers. Not only do spheroids comprise cells growing in a spherical arrangement, but they also contain extracellular matrices [19, 28] and develop pH, pO_2 and other metabolic gradients that create a micro-enviroment very similar to the acidic and hypoxic areas in solid tumour nodules [31]. They consist of an outer layer of proliferating cells and an inner layer of mainly quiescent cells, with massive necrosis often occurring in the central areas [5, 17]. This growth pattern is similar to that of many solid tumour nodules in vivo, which contain proliferating cells close to the capillaries, quiescent cells next to these and necrosis at larger distances. Their micro-environment and cell-cycle distributions, as well as possible penetration limitations, influence the effects of cytostatic drugs [11]. The two types of spheroids used in our experiments were chosen because they have been well characterised. The glioma U-118 MG spheroids exhibited a viable rim measuring about 300 µm, and that of the colon carcinoma spheroids measured about 200 µm. The glioma spheroids grew slower and displayed higher pO_2 and lower pH values in their inner regions than did the colon carcinoma spheroids [1, 2, 33]. These differences have been purported to be related to differences in glucose metabolism [2].

The histological method based on freeze-drying and vapour fixation has previously been described by Nederman et al. [27] for the application of cytostatic drugs and by Carlsson et al. [6] for the use of monoclonal antibodies. The method is used to preserve the distribution of both non-bound and bound drug. Sections of spheroids are processed for "dry" autoradiography to determine the spatial distribution of the radioactive test drugs. Accumulation can be seen on the autoradiographs as areas of high granular density. The average drug concentration in the spheroids was analysed by liquid scintillation counting following oil centrifugation for rapid separation of the spheroids from the drug-containing incubation medium.

Materials and methods

Spheroid cultures. Two cell lines, U-118 MG human glioma [37] and HT-29 human colon adenocarcinoma (American Type Culture Collection, Rockville, Md.; ATCC HTB 38), were cultivated as spheroids using the liquid overlay technique [4]. They were grown in Ham's F-10 culture medium supplemented with 10% fetal calf serum, L-glutamine (2 mM), streptomycin (100 µM/ml) and penicillin (100 IU/ml). Spheroids whose diameter measured 600 ± 100 µm were used in the experiments; such spheroids were obtained after about 5 weeks' culture.

Cytostatic drugs. [^{3}H]-Actinomycin D (481 GBq/mmol), [^{14}C]-daunomycin (1.67 GBq/mmol) and [^{3}H]-cytosine arabinoside (ara-C; 110 and 414 GBq/mmol) were obtained from Amersham (Sweden AB, Sweden). [^{14}]-Adriamycin (1.98 GBq/mmol) was supplied by Farmitalia, Carlo-Erba (Italy) and [^{3}H]-daunomycin (126 GBq/mmol) was obtained from New England Nuclear (FRG). The drugs were dissolved in normal culture medium prior to their use.

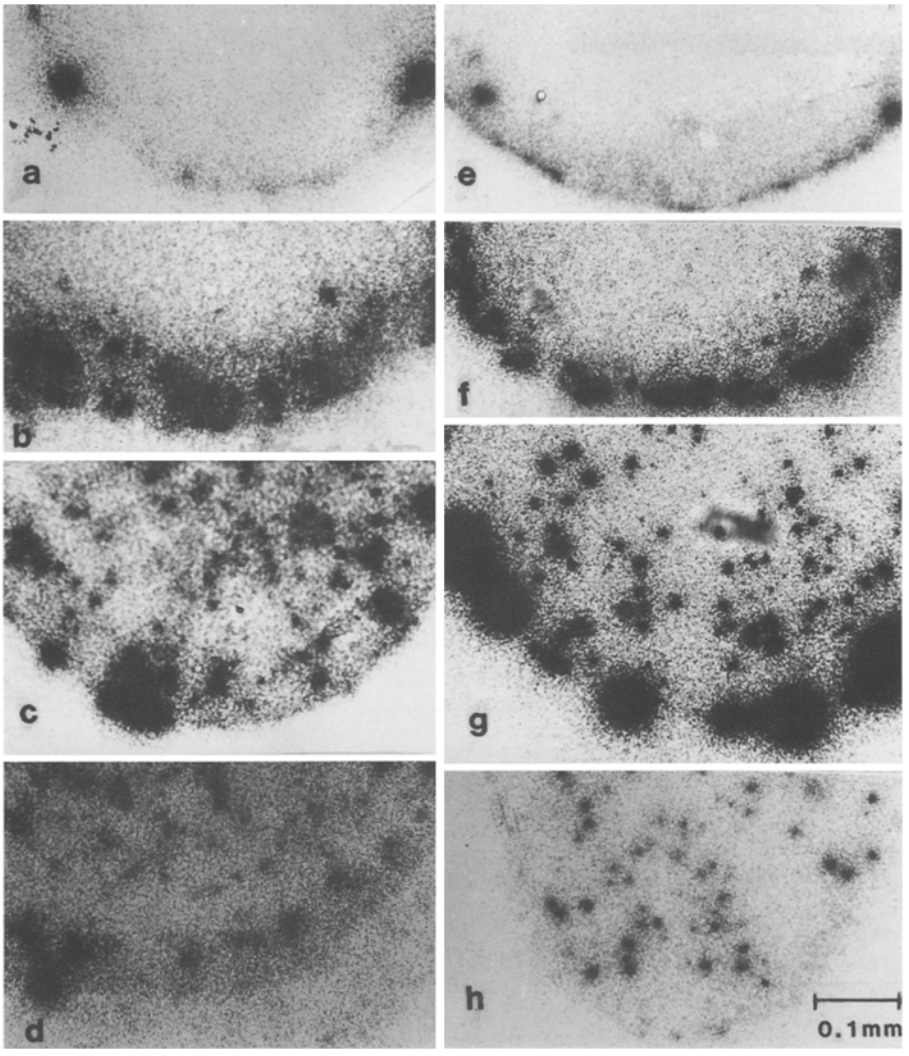

Fig. 1 a–h. Examples of contact autoradiograms showing the penetration patterns of [^{14}C]-Adriamycin in HT-29 (*left*, **a–d**) and U-118 MG (*right*, **e–h**) spheroids. The incubation times were **a** 1 min, **b** 15 min, **c** 60 min and **d** 19 min followed by washing for 3 × 20 s and incubation in drug-free medium and **e** 1 min, **f** 15 min, **g** 30 min and **h** 19 min followed by washing for 3 × 20 s and incubation in drug-free medium

Penetration studies. The penetration assay was designed to enable the detection on autoradiographs of all radioactive test substances that had penetrated into the tumour mass. The spatial distributions of both bound and non-bound test substances were preserved in the sections. The method has been described in detail elsewhere [6, 27] and is therefore only briefly discussed below.

The HT-29 and U-118 MG spheroids were incubated with 1 MBq of the tritiated drugs/ml and with 0.5 MBq of the [^{14}C]-labeled drugs/ml (Table 1). Only tracer amounts of the drugs were used to minimise toxicity. The period of incubation ranged from 30 to 60 min. The spheroids were thereafter placed on cover slips, frozen in propane cooled by liquid nitrogen, freeze-dried at –80° C (TIS-U-DRY, FTS Systems Inc., Stone Ridge, NY, USA) and vapour-fixed with paraformic aldehyde at 80° C. Finally, the spheroids were infiltrated with xylene, embedded in Histowax (Histolab, Sweden) and sectioned to 5 µm. The sections were dry-mounted on microscope slides and processed for contact autoradiography. With contact between the specimen and the hydrophilic solvent being avoided, a microscope slide with dried photoemulsion (Ilford K5) was placed on each section for an exposure period of 4 weeks. The slide with the photoemulsion was then removed, developed (Kodak D 19) and fixed.

Drug-retention test. The spheroids were incubated for 15 or 19 min with the labeled drugs as described above. Thereafter, they were rinsed for 3×20 s with drug-free culture medium and incubated for a further 15-min period in drug-free culture medium at 37° C. Finally, they were processed for the penetration assay as described above.

Grain counting. Drug penetration was quantitatively evaluated in sections from the penetration assay. Grain counting was done in a Leitz Orthoplan interference-contrast microscope. A circular grid placed in one of the 10× eyepieces was used in combination with a 100× interference-contrast objective to carry out stepwise radial evaluations of the local granular density on the autoradiographs. In the sections, the circular grid corresponded to a diameter of 7.2 µm (area, 40.7 µm^2). Background counts were evaluated at ten points lying 300 µm outside the spheroid surface. Counts were made at 0–10 µm inside the spheroids (position 0) and at depths of 50, 100, 200 and 300 µm. At least ten areas from two spheroid sections (from two different spheroids) were counted at each position. The granular density was renormalised to the number of grains in an area of 100 µm^2. Mean values and standard deviations were calculated.

Grain counting was done in selected areas in which the granular density was low so as to distinguish between penetration and accumulation. Accumulation was visible in certain areas, and the heterogeneous granular distribution seen in Figs. 1 (a–h), 3 (d–f) and 5 (a–h) probably depended on the intracellular accumulation (possibly via intercalation in DNA) of these drugs. In these cases, the evaluations were done "in between the accumulation areas". For daunomycin and ara-C in HT-29 spheroids and for ara-C in both types of spheroids, grain counting was done with no special area selection because the granular distribution was homogeneous in these cases. The peripheral regions of HT-29 spheroids that had been incubated with ara-C exhibited such high granular densities that it was not possible to obtain accurate counts.

Reference microscales for tritium (Amersham, RPA 501) and ^{14}C (Amersham, RPA 504) were used to estimate the granular density that

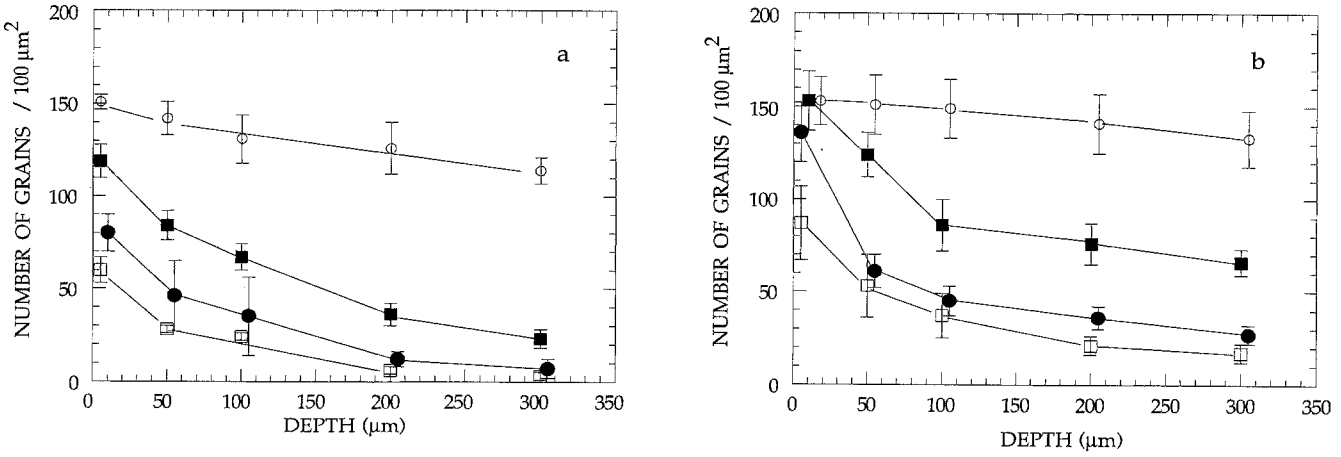

Fig. 2 a, b. Granular density in between the accumulation areas as a function of depth in **a** HT-29 and **b** U-118 MG spheroids after different periods of incubation with [^{14}C]-Adriamycin. □, 5 min; ●, 15 min; ■, 30 min; ○, 60 min

would be expected if the concentration of radioactivity in the spheroids were the same as that in the culture medium. The microscales containing tritium were purchased as blocks that were then sectioned to the same thickness as the sections in the penetration assay (5 µm) with a glass knife. The microscales containing ^{14}C were purchased as 120-µm-thick strips, which were then embedded in plastic and sectioned with a glass knife to 5 µm. The sectioned microscales were processed for contact autoradiography in the same manner as the spheroid sections in the penetration assay.

The concentrations of radioactivity in the microscales were given by Amersham as both the polymer activity and the corresponding tissue-equivalent values. We used the tissue-equivalent values. In our autoradiographical procedure, the sectioned ^{14}C microscale yielded about 36 ± 4 grains/100 µm^2 for a tissue-equivalent radioactive concentration of 0.5 MBq/g. This concentration corresponded to the ^{14}C concentration of 0.5 MBq/ml applied in the culture medium during the penetration tests. Thus, if the mean concentration of ^{14}C in the spheroids were the same as that in the culture medium, the granular density in the spheroid sections would lie in the range of 30–40 grains/100 µm^2. For the tritium microscale, 27 ± 3 grains/100 µm^2 corresponded to the tissue-equivalent radioactive concentration of 1.0 MBq/g. A tritium concentration of 1.0 MBq/ml was used in the culture medium during the penetration tests. Thus, if the mean concentration of tritium in the spheroids were the same as that in the culture medium, the granular density in the spheroid sections would lie in the range of 25–30 grains/100 µm^2.

Exact comparisons between the microscale values and the granular densities found in the spheroids cannot be made because of the difficulties in estimating the volumetric changes induced in the spheroid tissue by the histological procedures used (e. g. freeze-drying, vapour fixation). However, as the magnitude of this was probably similar in all cases, comparisons between different spheroid-drug combinations may never theless be possible.

Conventional histology and autoradiography. Following the incubation of spheroids with actinomycin D, we conducted conventional histology to visualise the intracellular binding of drugs in the accumulation areas. Drug and radioactivity concentrations were the same as those in the penetration studies (Table 1). The spheroids were incubated for 15 min, washed in culture medium, fixed in 10% neutral buffered formalin solution and embedded in glycolmethacrylate. Central sections measuring 2 µm in thickness were cut and autoradiography was performed. The slides were immersed in photoemulsion (Ilford K5) and air-dried. After 5 weeks they were developed (Kodak 19), fixed and stained with Mayer's hematoxylin.

Oil centrifugation and liquid scintillation counting. The average drug concentration in each spheroid was measured using a modification of the oil-centrifugation method described by Freyer and Sutherland [18]. Single spheroids were placed in microwells containing 0.2 ml medium. The spheroid volumes were calculated from the equation $V = 4/3\ \pi\ (a \times b)^{3/2}$, where a and b represent the observed minimal and maximal radii measured at right angles, respectively. The spheroids were incubated with the radioactive drugs (concentrations are given in Table 1) for different intervals ranging from 5 to 75 min. Following incubation, each spheroid together with the radioactive medium in the well was placed on top of 1 ml oil in a 1.5-ml centrifuge tube. The oil was a mixture of 1/6 corn oil (Mazola), 4/6 dibutylphthalate (Sigma D-2270) and 1/6 silicon oil DC 550 (18 270-500, Kebo-Lab, Stockholm, Sweden; recipe from Prof. R. M. Sutherland, personal communication). The tube was immediately spun in an Eppendorf centrifuge at 2000 rpm for 2 min to separate the spheroids from the radioactive medium. After the bottom of the tubes had been cut, single spheroids were solubilised with 1 ml Biolute-S (Zinsser Analytic, Frankfurt, FRG) and neutralised and the radioactivity was measured using 10 ml liquid scintillation cocktail (Quickscint-1, Zinsser Analytic, Frankfurt, FRG). Similar centrifugation procedures were used for controls, with spheroids being added to the radioactive solution on top of the oil mixture immediately prior to centrifugation (without incubation). Values amounting to about 2–5 times the normal background level in a liquid scintillation counter were measured in the controls, and these values were subtracted in calculations of the spheroid-associated radioactivity. The corrected radioactivity value for each spheroid was subsequently divided by the corresponding spheroid volume to obtain the mean value for the radioactive concentration in the spheroid. This value was finally divided by the concentration of radioactivity in the incubation medium (Table 1). The unit obtained was termed the relative radioactivity concentration (RRC), with 1 RRC unit corresponding to the concentration of radioactivity in the culture medium.

Results

Penetration studies

Adriamycin. The penetration pattern for [^{14}C]-Adriamycin was similar in HT-29 and U-118 MG spheroids (Fig. 1). After 1 min, the drug accumulated in a few areas near the spheroid surfaces (Fig. 1 a, e). By 15 min it had penetrated deeper and the accumulation areas were more frequently observed (Fig. 1 b, f). After longer incubation periods (30 or 60 min), accumulation areas could also be visualised in the central parts of the spheroids (Fig. 1 c, g). The drug-retention test, in which the spheroids were washed and incubated with drug-free medium, was performed after 19 min incubation with [^{14}C]-Adriamycin. Both HT-29 and U-118 spheroids retained the drug (Fig. 1 d, h) and the accumula-

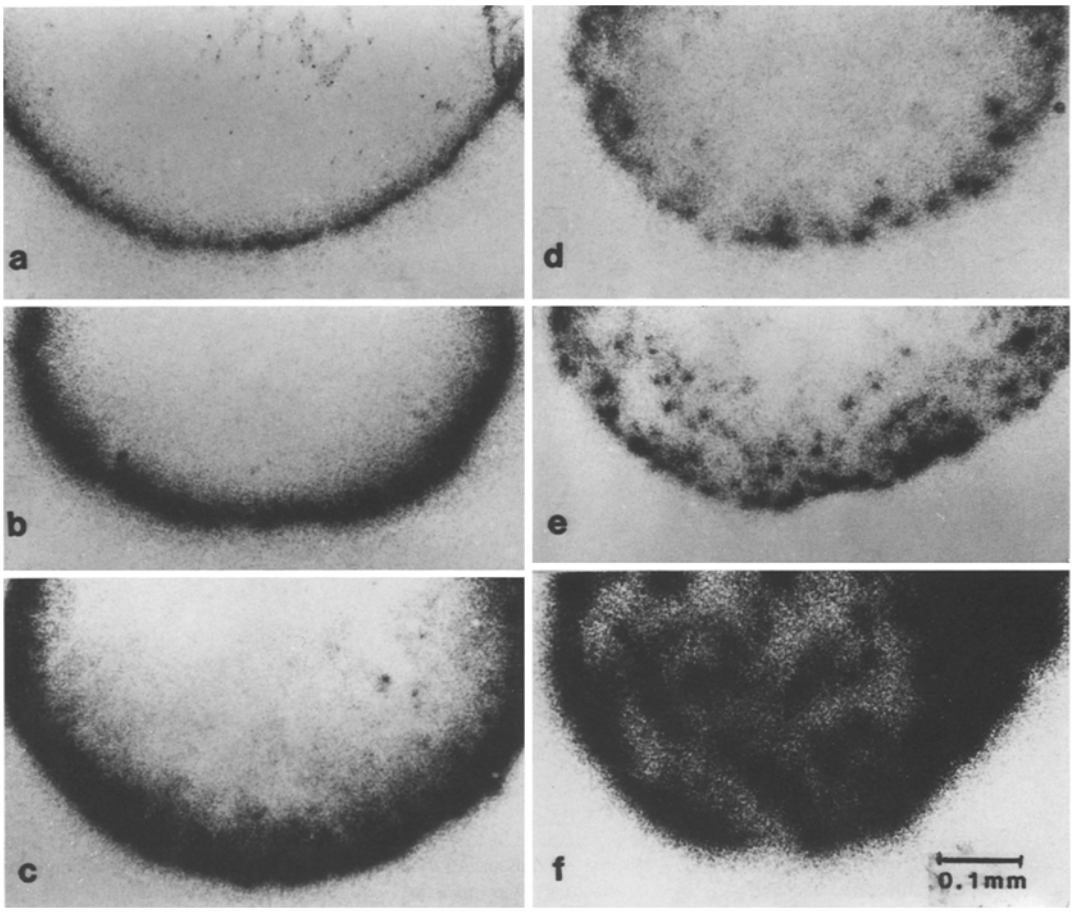

Fig. 3 a–f. Examples of contact autoradiograms showing the penetration patterns of [^{14}C]-daunomycin in HT-29 (*left*, **a–c**) and U-118 MG (*right*, **d–f**) spheroids. The incubation times were **a** 5 min, **b** 15 min, **c** 60 min, **d** 5 min, **e** 15 min and **f** 60 min

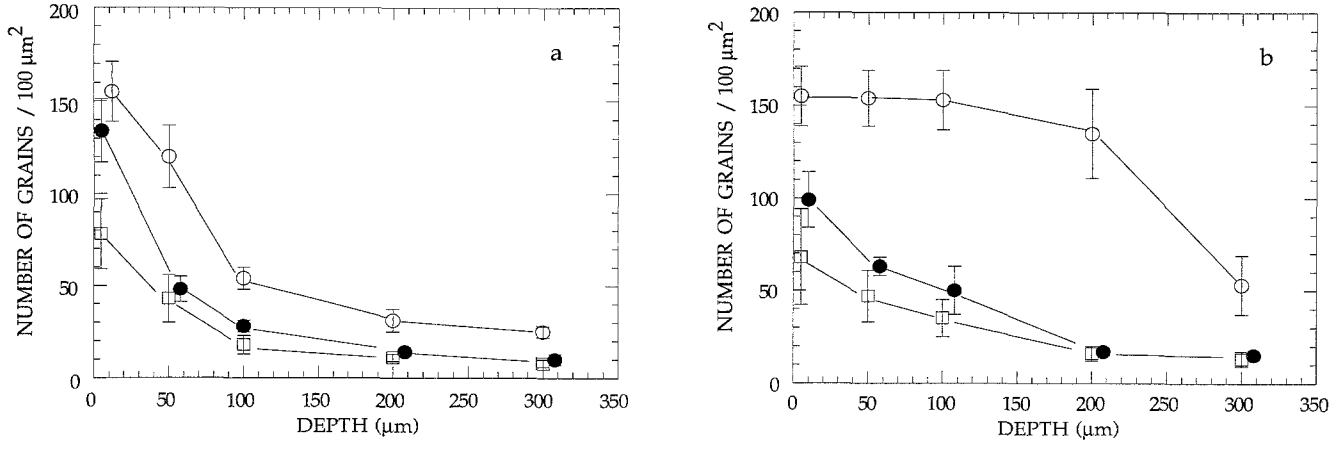

Fig. 4 a, b. Granular density in between the accumulation areas as a function of depth in **a** HT-29 and **b** U-118 MG spheroids after different periods of incubation with [^{14}C]-daunomycin. □, 5 min; ●, 15 min; ○, 60 min

tion areas remained, although the size of the latter decreaased in the U-118 MG spheroids.

Grain counting in between the accumulation areas revealed that Adriamycin penetrated well within 30 and 15 min in HT-29 and U-118 MG spheroids, respectively (Fig. 2). The ^{14}C reference scale indicated that a level of about 30–40 grains/100 µm^2 corresponded to the activity in the culture medium. At a depth of 200–300 µm, this level was achieved after 30- and 15-min incubations for HT-29 and U-118 MG spheroids, respectively. Lower central levels were seen following incubation periods of ≤15 min. Grain counting in the drug-retention test demonstrated decreased values only in the peripheral regions. The difference in shape between the 5-, 15- and 30-min curves and the 60-min curves in Fig. 2 was attributable to the finding that large amounts of the drug had penetrated into

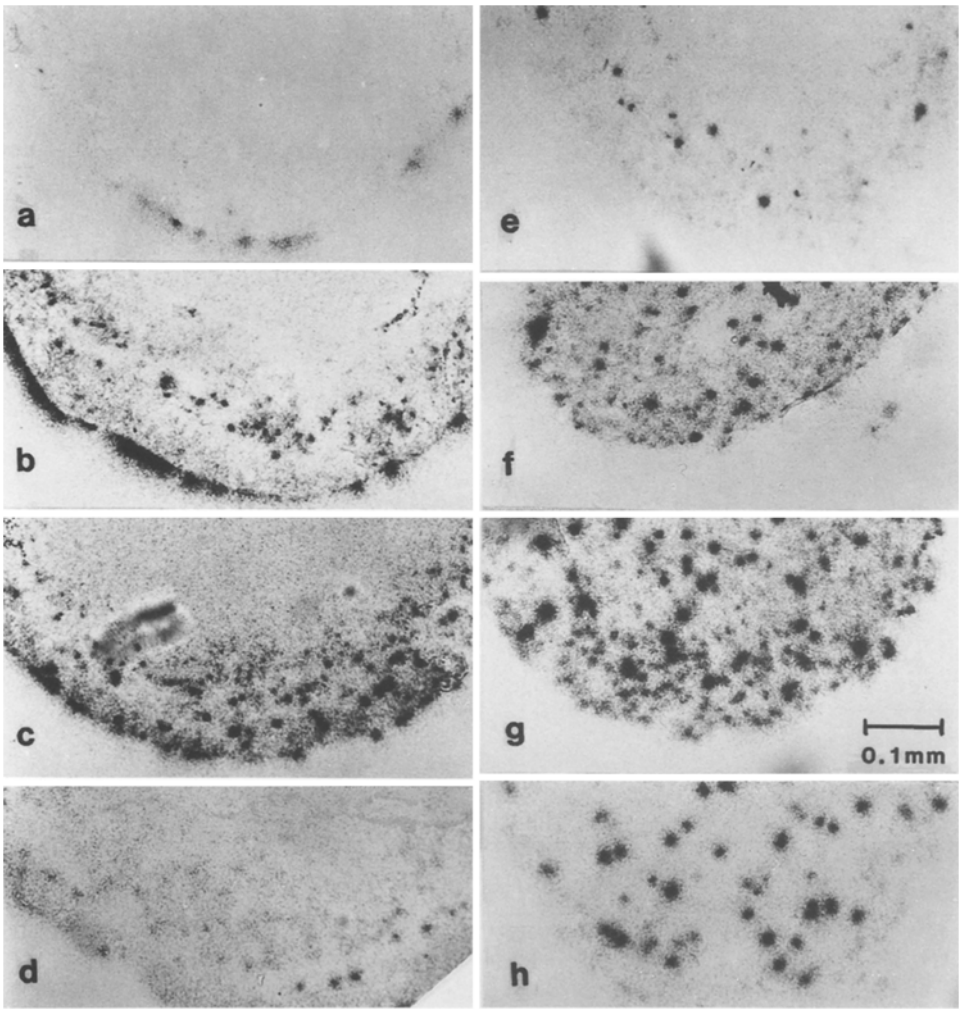

Fig. 5 a–h. Examples of contact autoradiograms showing the penetration of [^{3}H]-actinomycin D in HT-29 (*left*, **a–d**) and U-118 MG (*right*, **e–h**) spheroids. The incubation times were **a** 30 s, **b** 15 min, **c** 30 min and **d** 15 min followed by washing for 3 × 20 s and incubation for 15 min in drug-free medium and **e** 2 min, **f** 15 min, **g** 60 min and **h** 15 min followed by washing for 3 × 20 s and incubation for 15 min in drug-free medium

the deeper regions of the spheroids by 60 min, whereas this was not found to be the case after the shorter incubation periods.

Daunomycin. [^{14}C]-Daunomycin attached to the outer surface of HT-29 spheroids after 5 min incubation (Fig. 3a). After longer incubation periods (15 and 60 min), penetration into the central areas remained very poor and the drug was mainly observed in the cell layers near the spheroid surface (Fig. 3b, c). The peripheral accumulation of daunomycin was rather homogeneous, and no heterogeneous areas along the periphery such as those seen for Adriamycin in Figs. 1a–d could be found in the HT-29 spheroids. The penetration pattern of daunomycin in the glioma U-118 MG spheroids differed somewhat from that seen in the HT-29 spheroids. The drug penetrated into the glioma spheroids and heterogeneous grain accumulation was seen near the surface after 5 min incubation (Fig. 3d). After 15 min incubation, more cells accumulated the drug and the granular pattern could also be seen in deeper parts of the spheroids (Fig. 3e). After 60 min incubation, penetration seemed to be good and several accumulation areas were observed (Fig. 3f). A similar penetration pattern was obtained using [^{3}H]-daunomycin (data not shown).

Grain counting confirmed that daunomycin penetration was slow in the HT-29 spheroids (Fig. 4a), whereas that in the glioma U-118 MG spheroids seemed faster (Fig. 4b). Grain counting in the U-118 MG spheroids was done in between the accumulation areas. The ^{14}C reference scale indicated that a level of about 30–40 grains/100 μm^2 corresponded to the activity in the culture medium. This level was exceeded at a depth of 200–300 μm after 60 min incubation in U-118 MG spheroids and was nearly reached in HT-29 spheroids.

Actinomycin D. Only small amounts of [^{3}H]-actinomycin D were seen at the HT-29 spheroid surfaces after 30 s incubation (Fig. 5a). Several accumulation areas were observed in deeper regions after 15 and 30 min incubation (Fig. 5b, c). Actinomycin D penetrated rather well in the U-118 MG spheroids; as early as after a 2-min incubation period, it had accumulated in some cells located nearly 100 μm inside the spheroids (Fig. 5e). After 15- and 60-min incubation periods, more drug was taken up and the number of accumulation areas increased (Fig. 5f, g). The retention of [^{3}H]-actinomycin D was investigated after 15 min incubation followed by washing and incubation in drug-free medium. Because of disappearance of grains from the accumulation areas, there seemed to be some

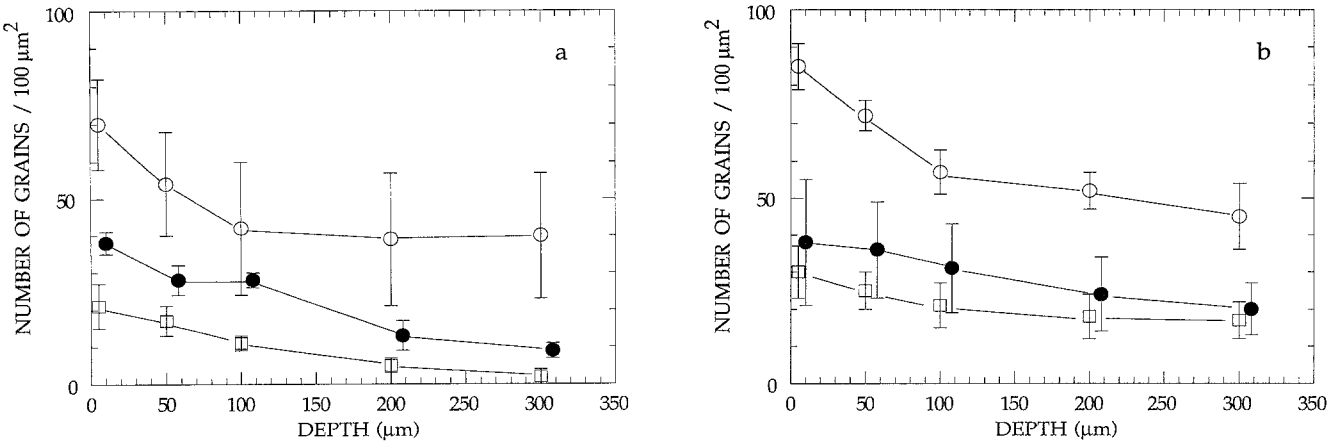

Fig. 6 a, b. Granular density in between the accumulation areas as a function of depth in **a** HT-29 and **b** U-118 MG spheroids after different periods of incubation with [^{3}H]-actinomycin D. □, 5 min; ●, 15 min; ○, 60 min

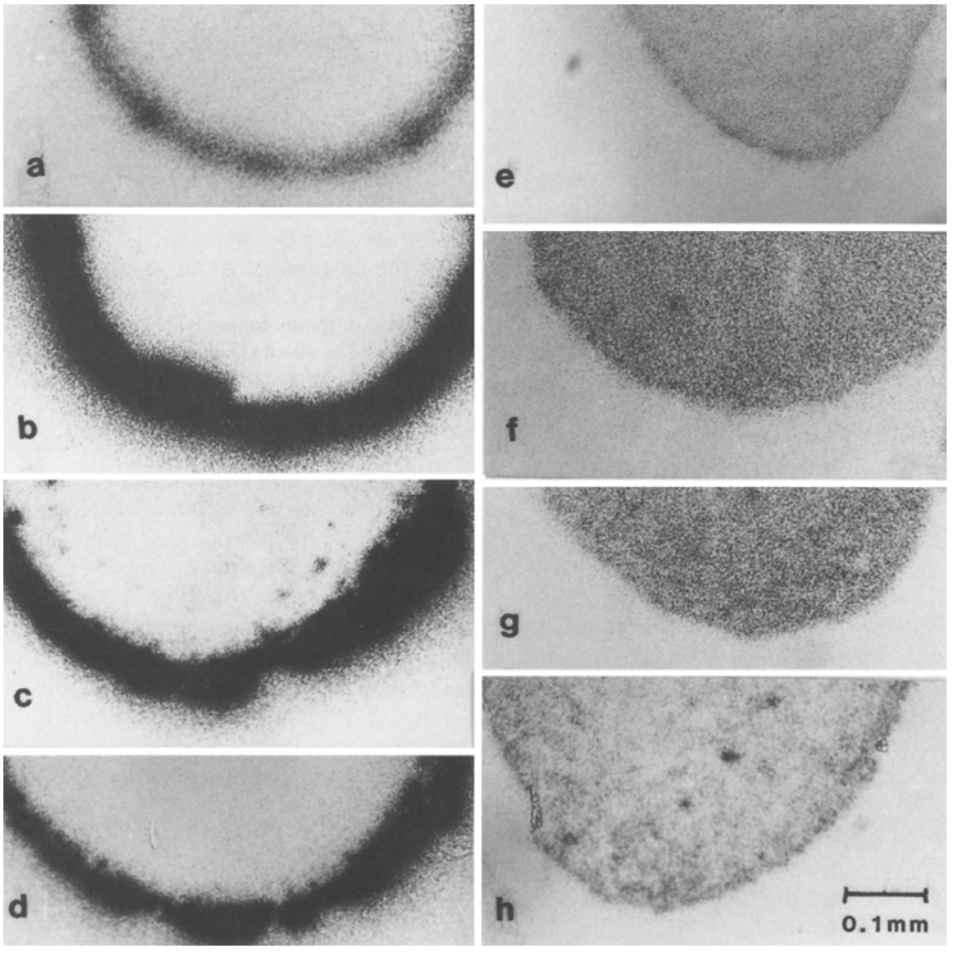

Fig. 7 a–h. Examples of contact autoradiograms showing the penetration patterns of [^{3}H]-ara-C in HT-29 (*left*, **a–d**) and U-118 MG (*right*, **e–h**) spheroids. The incubation times were **a** 2 min, **b** 15 min, **c** 30 min and **d** 15 min followed by washing for 3 × 20 s and incubation for 15 min in drug-free medium and **e** 2 min, **f** 15 min, **g** 30 min and **h** 15 min followed by washing for 3 × 20 s and incubation for 15 min in drug-free medium

washout in the HT-29 spheroids (Fig. 5d). In contrast, in the U-118 MG spheroids, the number of grains remaining in the granular areas indicated that the accumulated drug was more strongly bound (Fig. 5h).

Grain counting in between the accumulation areas demonstrated limited penetration of actinomycin D in both types of spheroid after 15 min but good penetration after 60 min (Fig. 6). The tritium reference scale indicated that a level of about 25–30 grains/100 µm^2 corresponded to the activity in the culture medium. The granular density decreased in all areas of both types of spheroid in the retention test.

Cytosine arabinoside. Cytosine arabinoside (ara-C) accumulated on the HT-29 spheroid surfaces as early as after a 2-min incubation period. The amount of surface-attached drug gradually increased with longer incubation periods, whereas penetration into the central areas seemed limited (Fig. 7a–c). In contrast, ara-C penetrated very efficiently in the U-118 MG spheroids and was homogeneously dis-

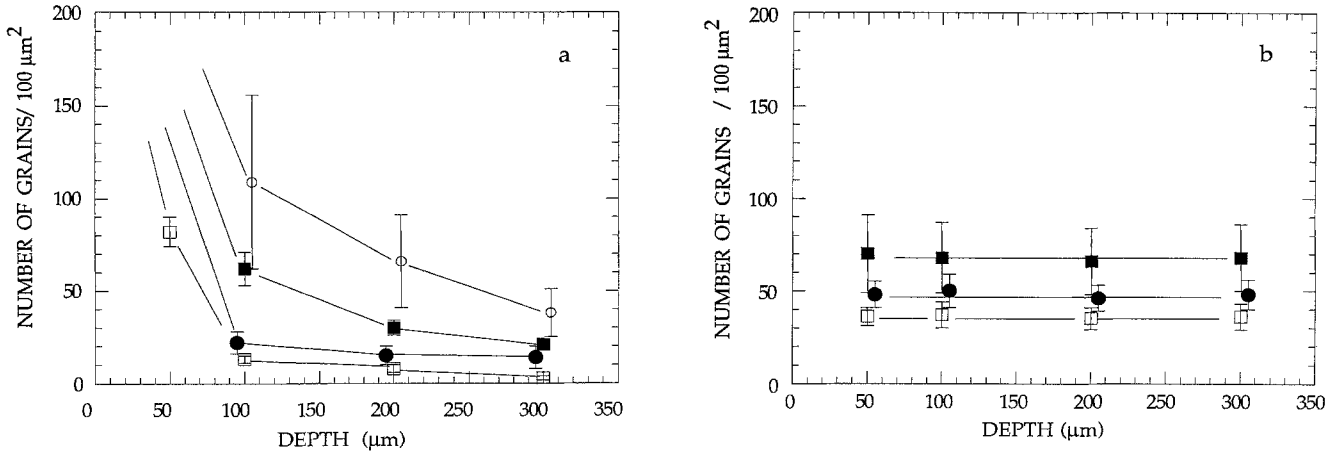

Fig. 8 a, b. Granular density in between the accumulation areas as a function of depth in **a** HT-29 and **b** U-118 MG spheroids after different periods of incubation with [^{3}H]-ara-C. □, 5 min; ●, 15 min; ■, 30 min; ○, 60 min

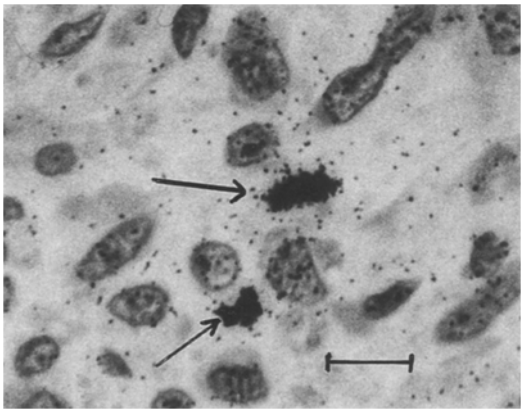

Fig. 9. Conventional autoradiogram of U-118 MG spheroid cells after 15 min exposure to [^{3}H]-actinomycin D. *Arrows* indicate intercalated nuclei. Bar = 10 µm

tributed within 2 min (Fig. 7e–g). Figure 7 shows the penetration patterns of [^{3}H]-ara-C of high specific activity (1.11 TBq/ml); no significant change was seen when the same radioactive concentration, albeit at a lower specific activity, was applied (0.414 TBq/ml, see Table 1; data not shown). Investigation of the drug retention by spheroids after 15 min incubation with [^{3}H]-ara-C followed by washing and 15 min incubation in drug-free medium revealed that the peripheral binding in the HT-29 spheroids resisted washing (Fig. 7d). To a large extent, the homogeneously distributed ara-C in U-118 spheroids was washed out (Fig. 7h).

Grain counting showed that ara-C penetrated into the central areas of the HT-29 spheroids after 60 min (but not ≤30 min), whereas its penetration into the U-118 MG spheroids was good as early as after 5 min (Fig. 8). The tritium reference scale indicated that a level of about 25–30 grains/100 µm^2 corresponded to equilibrium with the activity in the culture medium. The granular density decreased in all areas of the U-118 MG spheroids in the retention test.

Accumulation clusters

Following the incubation of spheroids with the intercalating drugs Adriamycin, daunomycin and actinomycin D, the radioactivity accumulated in smaller areas corresponding to single cells or to clusters of cells (Figs. 1, 3, 5). The accumulation clusters were smaller when tritium was used as the radiotracer. As the mean range of the electrons from tritium is about 1 µm, whereas that of ^{14}C electrons is about 100 µm, it is reasonable to assume that the clusters on the autoradiographs became smaller when tritiated drug was used.

Figure 9 shows the binding of [^{3}H]-actinomycin D to cell nuclei after 15 min incubation of a U-118 MG spheroid. This spheroid was processed for conventional histology and autoradiography, and the autoradiograph demonstrated labelling in some nuclei that may have corresponded to DNA untercalation. Thus, this type of binding probably gave rise to the granular accumulation areas seen in the penetration studies for all three intercalating agents. In the case of Adriamycin, it is likely that several cells formed the larger clusters. Using flow cytometry, Durand [8] and Durand and Olive [13] have previously found intracellular binding of Adriamycin in both spheroids and corresponding monolayers that was probably due to intercalation of the drug in DNA.

Oil centrifugation and liquid scintillation counting

The average drug concentrations in the HT-29 colon carcinoma and U-118 MG glioma spheroids were analysed using the oil-centrifugation method. Following centrifugation in oil for rapid separation of the spheroids from the surrounding medium, the spheroids were solubilised and the radioactivity was counted in a liquid scintillation counter.

The accumulation of [^{14}C]-Adriamycin began after short incubation periods in both types of spheroid and reached about 3 times that measured in the culture medium (3 RRC units) after 60–75 min (Fig. 10a). Two concentrations of radioactivity were tested (Table 1) but no signifi-

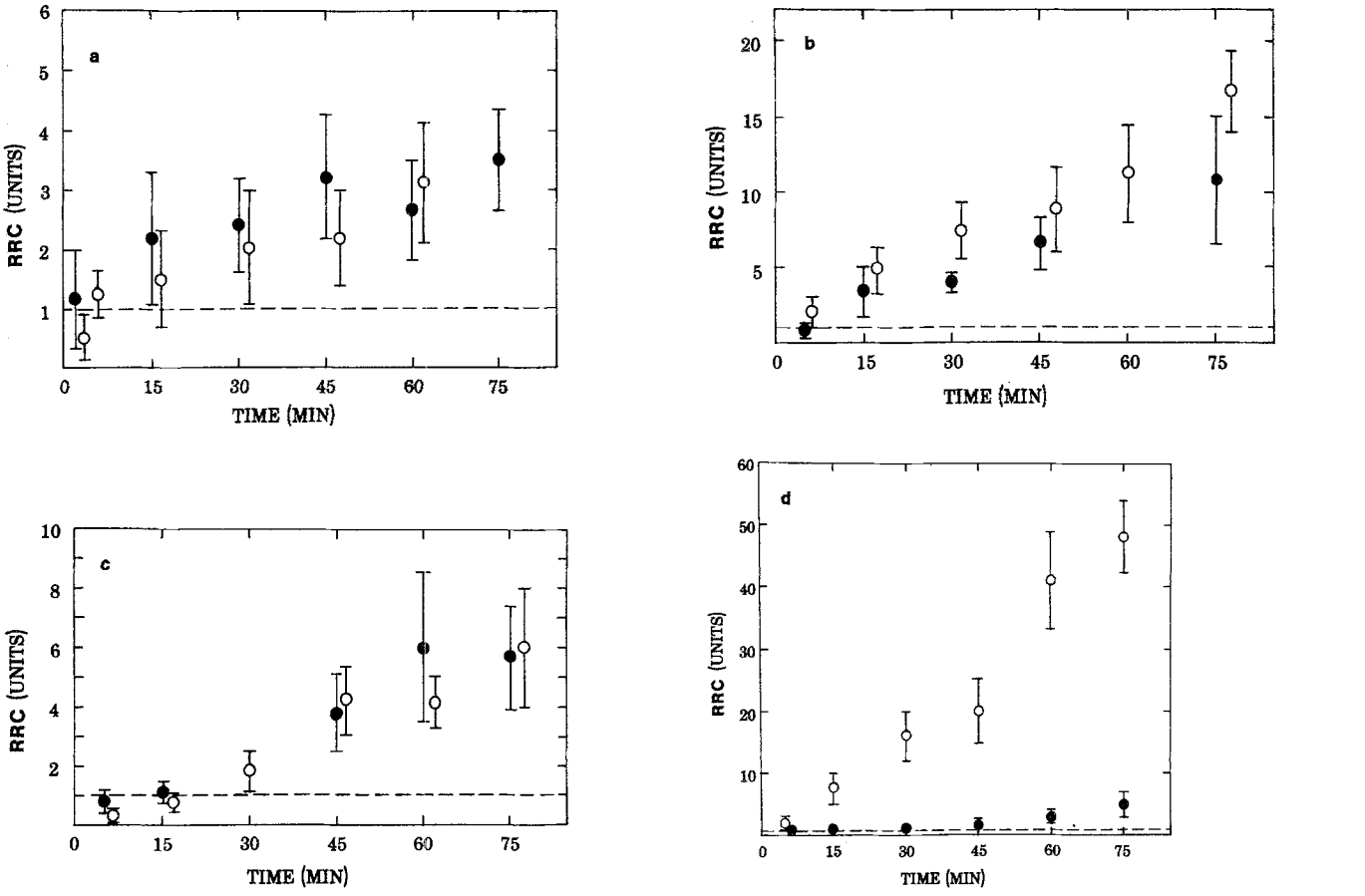

Fig. 10 a–d. Average drug concentrations associated with HT-29 (*open circles*) and U-118 MG (*filled circles*) spheroids at different times after incubation with the radioactive drugs **a** [^{14}C]-Adriamycin, **b** [^{14}C]-daunomycin, **c** [^{3}H]-actinomycin D and **d** [^{3}H]-ara-C. Mean values ± maximal deviations are shown

cant difference was found. The data shown in Fig. 10a were obtained following incubations with 0.10 MBq/ml. The amount of [^{14}C]-daunomycin accumulated after 75 min incubation corresponded to about 10 RRC units in U-118 MG spheroids and ca. 15 RRC units in HT-29 spheroids (Fig. 10b). This high accumulation in HT-29 spheroids must have been a consequence of attachment of the drug to the peripheral regions such as that observed in the penetration studies (Fig. 3c). A slowly increasing accumulation of [^{3}H]-actinomycin-D was observed in both types of spheroid (Fig. 10c). After a 15-min incubation period the drug concentration in the spheroids was equal to that in the medium, and after 75 min it was about 6 times higher (6 RRC units). The accumulation of [^{3}H]-ara-C was extremely high, reaching levels of up to 40–50 RRC units after 60–75 min in the HT-29 spheroids (Fig. 10d). The explanation for this is that large amounts of drug accumulated on the HT-29 spheroid surfaces (Fig. 7a–c). In the U-118 MG spheroids the accumulation of [^{3}H]-ara-C was only about 4 RRC units after a 75-min incubation despite the good penetration observed (Fig. 7e–g).

Discussion

In several studies, it has been proposed that the binding or toxic action of cytostatic drugs in the peripheral regions of tumour nodules or in the outer layers of tumour spheroids is a consequence of limited penetration [3, 9, 10, 15, 21, 22, 24, 26, 27, 31, 32, 36]. One interesting hypothesis is that binding prevents penetration; this was suggested by Durand [12] in a study on the toxic effects of Adriamycin on hamster V79 spheroids. Our idea is that tumour spheroids exhibit properties similar to those normally observed in affinity chromatography, whereby substances that undergo binding or other interactions display delayed penetration, if any, whereas substances that do not undergo binding can easily penetrate the spheroids. The results obtained in the present study support this idea.

Penetration was slow or strongly limited when drug accumulation was extensive and homogeneous in the peripheral regions of the spheroids. Daunomycin and ara-C in colon carcinoma HT-29 spheroids are examples in which the penetration of drug into the central areas of the spheroids was very slow, with about 1 h being required for the concentration in the central regions to reach that measured in the culture medium. Thus, these two cases seem to be examples of limited penetration due to peripheral binding.

Quite another situation was seen for ara-C penetration in the glioma U-118 MG spheroids. In this case, the penetration was so fast that equilibrium with the culture medium was obtained within 5 min. At the same time, there seemed to be very little binding, because the retention test showed that most of the drug had been washed out.

Furthermore, the mean concentration of drug in these spheroids was very low, at least after incubation periods of <1 h, as shown both by grain counting in sections and by the oil-centrifugation method. Thus, this seems to be an example of low binding that correlates with good penetration. There was no sign of intracellular ara-C accumulation in the contact autoradiograms of HT-29 and U-118 MG spheroids. Whether this may have been attributable to retarded membrane transport or to other mechanisms could not be judged using our methods.

A third case involved the occurrence of granular accumulation areas in the spheroids that indicated heterogeneous binding. Typical examples are Adriamycin and actinomycin D in glioma U-118 MG and colon carcinoma HT-29 spheroids and daunomycin in the glioma spheroids. In these cases the intercalating drugs seemed to bind only in certain areas. Except for actinomycin D in colon carcinoma spheroids, the retention test showed that the drug was retained in the accumulation areas. The retention test also revealed that the drug concentration decreased in regions located between the accumulation areas. This was most clearly seen after washing of the glioma spheroids as shown in Fig. 5 h. In addition to heterogeneous binding, common properties exhibited by daunomycin and actinomycin D included poor penetration into the central regions of the spheroids after 15 min incubation, although their penetration was good after 60 min. This phenomenon was classified as intermediately good penetration. Such a penetration pattern is in accordance with heterogeneous binding, since there are regions of non-binding through which the drugs can penetrate.

Thus, it seems that the penetration of substances that do not undergo binding is good. This finding is supported by the results of a previous study in which it was shown that substances such as thymidine-5′-triphosphate, sucrose and inulin, which cannot bind to cell membranes or to the extracellular matrix and cannot cross cell membranes, easily penetrate spheroids [29]. A similar result, along with limited binding and good penetration, has been reported for monoclonal anti-carcinoembryonic antigen (CEA) antibodies in colon carcinoma spheroids [6].

The results of the present study imply that good penetration does not necessarily result in a high average concentration of drug; this was the case for ara-C, which penetrated well in glioma spheroids but did not reach a high average concentration. In fact, the average concentration of both ara-C and daunomycin measured in colon carcinoma spheroids was higher than that achieved in glioma spheroids, despite their limited penetration into the former. Of course, this was due to the extensive accumulation of drug in the peripheral regions of the colon carcinoma spheroids.

Assuming that the relationships found between penetration and binding are also valid in vivo (in the clinical situation), the situation is obviously less than optimal if the accumulation of drug near vessels and capillaries is so massive and homogeneous that its penetration is prevented or severely delayed. In such cases, areas situated 100–200 μm from the vasculature cannot be reached by the drug. Thus, if the colon carcinoma HT-29 spheroids were typical for a given tumour in vivo, it seems that daunomycin and ara-C would not be effective in treating the latter. Of course, this example is only theoretical, and it is difficult to judge the extent to which such spheroids are representative of the clinical situation.

A less than optimal situation also arises when the drug is not taken up strongly in the tumour, as exemplified by ara-C in the glioma U-118 MG spheroids. In this case, we found quite good penetration but low average concentrations in the spheroids. An ideal setting might be achieved if the drug were to bind strongly in some areas so as to give a high average concentration in the tumour while simultaneously being capable of penetrating into the deeper areas of the tumour nodules. This seemed to be the case for Adriamycin and actinomycin D in the glioma U-118 MG and the colon carcinoma HT-29 spheroids and for daunomycin in the glioma spheroids. Durand [12] has recently pointed out that the binding of anthracyclines in some areas of a tumour and their delayed release might result in an improved therapeutic effect. This might well be the case for Adriamycin, because if the drug were released from the accumulation areas after a given interval, it could penetrate to other areas and exert additional toxic action. Thus, the beneficial effects of Adriamycin for the treatment of malignancies might well be dependent on its capacity both to bind and to penetrate the tumour.

It is noteworthy that Adriamycin and daunomycin exhibited different penetration properties (especially in the HT-29 spheroids) despite their close similarity in chemical structure. It is well known that their therapeutic efficacy in the treatment of solid tumours also differs whereby Adriamycin is generally more effective. Detailed studies of the penetration, binding and therapeutic effects of these drugs in spheroids could be of value in revealing the reasons for such differences. The penetration pattern of drugs such as Adriamycin, daunomycin and actinomycin D might also be influenced by the presence of multidrug transporter p-glycoproteins. However, we have not yet addressed the question as to whether HT-29 and U-118 MG cells express p-glycoproteins.

We plan to carry out further studies using spheroids in which the possibility of predicting the therapeutic effects of drugs by considering the results obtained in the present investigation will be analysed. Drug concentrations, incubation periods and AUC (area under the concentration-time curve) values could then be chosen according to the levels that are achievable in patients. In the present study we chose only concentrations that were suitable for the autoradiographical detection of the labelled drugs. In the judgement of therapeutic efficacy, it should also be borne in mind that the methods used in the present study detect the drug-associated radioactivity without distinguishing the native drug from the cleavage products. It is possible that active non-radioactive cleavage products might exhibit spatial distribution patterns in the tumour spheroids other than those observed by the applied techniques.

Further studies should also be conducted to reveal whether the penetration barrier observed in the case of ara-C is unique for colon carcinoma HT-29 spheroids or whether it also occurs in other types of colon carcinoma spheroid. Colon carcinomas are clinically resistant to many cytotoxic drugs, including ara-C [14]. In a previous study

using electron microscopy tight junctions and microvilli similar to those found in the epithelium of the colon [25] were seen on the surface of HT-29 spheroids. Cultured colon carcinoma cells are also known to excrete mucus [35], as do the epithelial cells in the intestinal tract. This mucus protects the cells against the contents of the bowel and may possibly also provide a penetration barrier to chemotherapeutic agents. This possibility should be analysed in more detail.

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