Distribution of disulfonated and tetrasulfonated aluminum phthalocyanine between malignant and host cell populations of a murine fibrosarcoma

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Abstract

Levels of disulfonated and tetrasulfonated aluminum phthalocyanines ($AlPcS_{2,4}$) were measured in cells derived from FsaR tumors (murine fibrosarcoma) using a fluorescence-activated cell sorter (FACS). The tumors were excised from animals injected with the sensitizer 24 h earlier and enzymatically dissociated. Before flow cytometry, the cells were stained with fluorescein isothiocyanate-conjugated anti-mouse monoclonal antibodies to specific immune cell membrane markers (Mac1, Fc receptor (FcR) or CD45). Staining to FcR and CD45 was combined with a DNA stain Hoechst 33342. This enabled concomitant discrimination to be made by the FACS between different populations of tumor-infiltrating host cells and malignant cells. The results showed on average 1.49 times higher $AlPcS_2$ levels and 1.16 times higher $AlPcS_4$ levels in Mac1-positive ($Mac1^+$) compared with Mac1-negative ($Mac1^-$) tumor cell populations. The same type of experiments performed with SCCVII tumor (squamous cell carcinoma) gave average $Mac1^+/Mac1^-$ ratios of 1.75 and 1.45 for $AlPcS_2$ and $AlPcS_4$ respectively. The data using other antibodies and DNA staining are consistent with the conclusion that, based on average per cell content, elevated levels of $AlPcS_2$, and to a lesser extent $AlPcS_4$, are retained in tumor-associated macrophages (TAM). The levels of these photosensitizers in other leukocytes and in non-immune host cells were not substantially different from those in malignant tumor cells. It is also shown that elevated levels of $AlPcS_2$ and $AlPcS_4$ are not localized in all TAM, but rather in a fraction of this cell population characterized by extremely high photosensitizer content.

Key words: Photosensitizers; Aluminum phthalocyanines; Photodynamic therapy; Tumor-infiltrating host cells; Flow cytometry

1. Introduction

One of the most important issues in photodynamic therapy (PDT) concerns the localization of photosensitizers in tumors and normal tissues. It has become evident that it is not accurate to claim that most photosensitizers have tumor-localizing capacity [1]. Normal tissues, including liver, kidney, bladder, spleen, pancreas, lymph nodes and skin, generally show photosensitizer levels higher or similar to those in tumors [1]. There is, however, some selectivity of photosensitizer retention in tumors compared with surrounding normal tissue; the difference in many cases does not exceed the ratio 2:1 [1–3]. There is no evidence to suggest that malignant cells in tumors possess, in general a capacity for preferential photosensitizer uptake. The elevated photosensitizer levels in tumors can rather be attributed to the accumulation of the drug in other structures, such as necrotic areas and tumor stromal elements [1, 4, 5]. We [6, 7] and others [5] have shown that tumor-associated macrophages (TAM) are capable of accumulating photosensitizer levels higher than in malignant tumor cells, which can contribute significantly to the selectivity of photosensitizer tumor retention.

In previous work, we have stained murine tumor-derived cell suspensions with fluorescein isothiocyanate-conjugated (FITC-conjugated) anti-mouse IgG to discriminate between cell populations which are Fc receptor (FcR) positive (more than 95% TAM) and FcR negative (mostly malignant tumor cells) and have measured Photofrin fluorescence in these different cell populations using a fluorescence-activated cell sorter (FACS) [6]. The measurements performed for the time intervals 1–96 h after Photofrin administration to mice bearing SCCVII squamous cell carcinoma have suggested

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consistently higher photosensitizer levels in TAM than in malignant tumor cells.

In this study, DNA staining with Hoechst 33342 and monoclonal antibodies to mouse IgG and to Mac1 and CD45 immune cell surface markers were used in conjunction with flow cytometry to measure the distribution of disulfonated and tetrasulfonated aluminum phthalocyanine ($AlPcS_2$ and $AlPcS_4$) in different cellular populations of a murine FsaR fibrosarcoma. In particular, an attempt was made to compare the levels of these two photosensitizers in four cell populations that are of special interest: malignant tumor cells, TAM, tumor-infiltrating leukocytes other than TAM and non-immune host cells (endothelial cells) in tumors.

2. Materials and methods

Tumors were grown in female C3H/HeN mice 9–11 weeks of age. Mouse fibrosarcoma FsaR [8] was obtained from Dr. G. Dougherty. It was maintained by biweekly intramuscular passage, by injecting the tumor brei (obtained from minced tumor tissue without enzymatic treatment) containing approximately 1×10^6 cells into the hind legs of Metofane-anesthetized animals. The same maintenance has been practiced in our laboratory for SCCVII tumor (squamous cell carcinoma) [9]. For experiments, the tumors were implanted subcutaneously, by injecting 3×10^5 cells in a 0.03 ml volume over the sacral region of the back of the anesthetized mice. Tumors were used when they had attained a size of 200–400 mg (wet weight).

The photosensitizers $AlPcS_2$ and $AlPcS_4$ were obtained from Porphyrin Products Inc. (Logan, UT). The $AlPcS_2$ preparation is a mixture of disulfonates at different positions on the phthalocyanine ring with traces of monosulfonated and trisulfonated derivatives. For stock solutions, the photosensitizers were dissolved in physiological saline at 2.5 mg ml^{-1}. They were administered to mice at 10 mg kg^{-1}, intravenously (i.v.), 24 h before sacrifice and tumor excision.

The excised tumors were minced and then enzymatically digested by a 30 min treatment with a mixture of 0.1% dispase (Boehringer, Mannheim, Germany), 0.02% collagenase (Sigma Chemical Co., St. Louis, MO) and 0.015% DNAse (Sigma) at 37 °C [10]. The single-cell suspensions obtained were resuspended in Eagle's MEM medium with 10% fetal bovine serum (FBS) (all from HyClone Laboratories Inc., Logan, UT) and aliquots were divided for different staining procedures. The viability of the cells was always over 75%. The enzymatic digestion procedure used is not selectively detrimental to tumor-infiltrating macrophages and lymphocytes [11]; it had no detectable effect on the cell viability and the expression of the cell membrane markers analyzed. Cells were stained for Mac1 using supernatants from the hybridoma cell line (ATCC TIB128) producing rat antimouse monoclonal antibodies directed against this antigen following the procedure described by McBride *et al.* [12]. The hybridoma supernatant (0.15 ml) was added to the pellet of 1×10^6 cells and kept on ice (0 °C) for 45 min. The cells were then centrifuged and washed twice in Hank's balanced salt solution (HBSS) with 2% FBS. This was followed by staining with 0.15 ml of FITC-conjugated goat anti-rat IgG F(ab')2 fragment (Cedarline, Ont.) diluted 1:1000 in HBSS + 2% FBS. After 30 min incubation on ice, the cells were washed twice in HBSS + 2% FBS and analyzed by flow cytometry using a dual laser apparatus (FACS 440, Becton-Dickinson) with three independent photodetectors. The cells were simultaneously divided into $Mac1^+$ and $Mac1^-$ populations (FITC bright and FITC dim fluorescence respectively) and analyzed for intensity of $AlPcS_2$ and $AlPcS_4$ fluorescence. Fluorescein was excited by the 488 nm laser and the emission was measured through a 530 ± 15 nm bandpass filter. The photosensitizer was excited by the UV laser (350–360 nm) and emission over 635 nm was recorded using a longpass filter. Dead cells, erythrocytes and debris were gated out using the forward light scatter analysis.

Staining with FITC-conjugated goat anti-mouse IgG (whole molecule) (Sigma) has been described previously [6]. Briefly, FITC-IgG was added to the cell suspension at 1:200 final dilution (v/v), incubated for 1–2 min at 37 °C, centrifuged (5 min), washed and processed immediately by flow cytometry. Staining for CD45 antigen was performed using monoclonal antibody YE1.21 generously provided by Dr. F. Takei. The procedure was identical to staining for Mac1 antigen, including the use of the FITC-conjugated secondary antibody.

In this work, staining for FcR and CD45 antigens was preceded by staining with a fluorescent DNA dye Hoechst 33342 [9] purchased from Sigma. Hoechst 33342 solution (1 ml) (8.43 μg ml^{-1}) in physiological saline was added to the cell pellet and placed in a 37 °C water bath for 75 min. The unbound dye was then removed by centrifugation and washed twice in HBSS + 2% FBS. During FACS analysis, Hoechst 33342 was excited by the UV laser and its fluorescence was recorded using

a 449 ± 5 nm bandpass filter. Approximately 10 000 cells were examined for every FACS analysis.

3. Results and discussion

3.1. Staining for Mac1 antigen

Tumor-bearing mice were given $AlPcS_2$ or $AlPcS_4$ (10 mg kg^{-1}, i.v.) and sacrified 24 h later. The tumors were excised and disaggregated by enzymatic treatment. Single-cell suspensions obtained in this way were stained with a rat monoclonal antibody to mouse Mac1 and then with FITC-conjugated anti-rat IgG, as described in Section 2. When examined for FITC fluorescence under flow cytometry, the cells from these suspensions can be clearly separated into two populations: brightly fluorescing ($Mac1^+$) and dimly fluorescing ($Mac1^-$). The $Mac1^+$ population should consist of cells characterized by the presence of Mac1 antigen, *i.e.* macrophages and granulocytes. Since the incidence of granulocytes in the tumors used in this study (FsaR and SCCVII) is very low compared with the macrophage content, it can be assumed that the $Mac1^+$ population fairly represents the TAM population of these tumors. The $Mac1^-$ population should include all the other cells present in these tumors, *i.e.* predominantly malignant tumor cells, but also the other host immune and non-immune cells.

A characteristic flow cytogram for gated $Mac1^+$ and $Mac1^-$ tumor cell populations from a mouse administered $AlPcS_2$ is shown in Fig. 1. It can be seen that most of the $Mac1^-$ cells, over five times more abundant than the $Mac1^+$ cells, are grouped in the region of low intensity of fluorescence in the red. The $Mac1^+$ cells are much more heterogeneous, showing a wide distribution of red fluorescence intensity; 11% of these cells were distributed in the overflow (last four channels at far right), *i.e.* exhibited a very high intensity of photosensitizer fluorescence. Very similar flow cytograms were obtained with $AlPcS_4$ (not shown).

The photosensitizer fluorescence distribution within $Mac1^+$ and $Mac1^-$ populations was further analyzed by dividing these two populations from individual tumors into ten fractions, each consisting of 10% of the total respective population (Fig. 2). Fraction 1 is formed from 10% of the analyzed cells showing the lowest intensity of red fluorescence in a given population, while fraction 10 contains 10% of the cells exhibiting the highest red fluorescence intensity. The data shown are average values for the ten fractions from a representative experiment with four tumors per group.

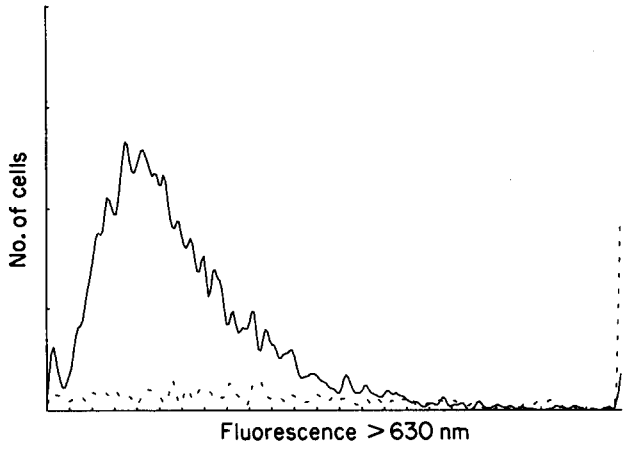

Fig. 1. Flow cytogram showing the distribution of $AlPcS_2$ in $Mac1^+$ and $Mac1^-$ cells from an FsaR tumor. The photosensitizer (10 mg kg^{-1}, i.v.) was administered 24 h before the tumor was excised, enzymatically digested and cells stained for Mac1 antigen. The ordinate (number of cells) and abscissa (intensity of fluorescence at greater than 630 nm in arbitrary units distributed in 256 channels) are on a linear scale.

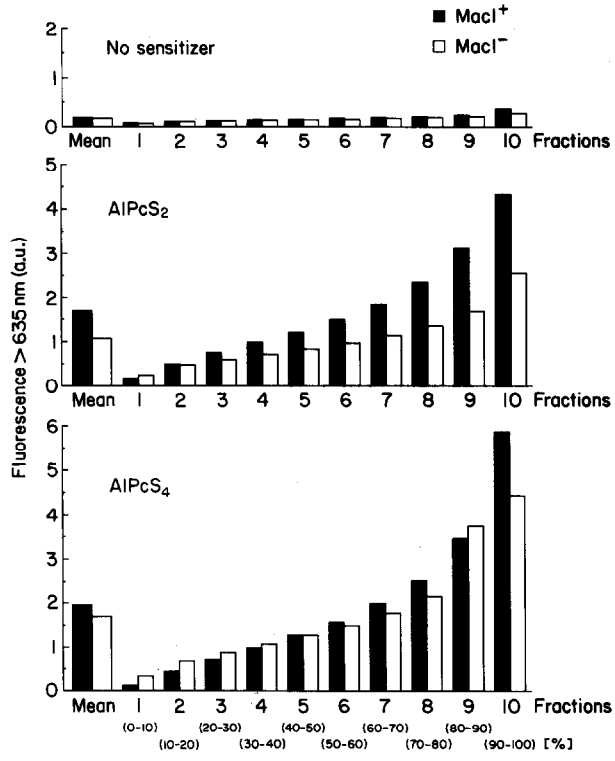

Fig. 2. Distribution of fluorescence intensities in the region greater than 635 nm measured by a FACS for the cells in $Mac1^+$ and $Mac1^-$ populations obtained from FsaR tumors excised from mice receiving no photosensitizer or aluminum phthalocyanines (10 mg kg^{-1}, i.v.) 24 h before tumor excision. Cell populations were divided into ten fractions (each containing 10% total cell population) with increasing fluorescence intensities of greater than 635 nm. Standard deviations for each column were no greater than 10%.

The mean values (including all of the ten fractions) indicate that Mac1$^+$ populations are characterized by higher levels of photosensitizer fluorescence for both $AlPcS_2$ and $AlPcS_4$, although with using $AlPcS_4$ the difference between Mac1$^+$ and Mac1$^-$ is lower. However, fraction 1 in Mac1$^+$ populations from tumors containing $AlPcS_2$ shows a ten times lower photosensitizer fluorescence than the mean value of the whole population, and no higher than the average "background red fluorescence" in Mac1$^+$ cells from tumors with no photosensitizer administered (also shown in Fig. 1). At the other extreme, the photosensitizer fluorescence in fraction 10 was 2.5 times higher than the mean for Mac1$^+$. A similar distribution, indicating a high heterogeneity of photosensitizer levels for Mac1$^+$ cells, is seen with $AlPcS_4$.

The values for "background red fluorescence", shown in Fig. 2 as the data from tumors excised from animals not given the photosensitizer, were not subtracted from the photosensitizer fluorescence values. This low-level "background red fluorescence" evidently varies considerably in different types of cells found in an FsaR tumor, and there is an inherent problem with mouse tumor-to-tumor heterogeneity. The experience in FACS analysis suggests that subtraction of such values from photosensitizer fluorescence can lead to inaccuracy in the results. Tumor-to-tumor heterogeneity in overall photosensitizer levels also occurs. Arbitrary units (a.u.), the units of fluorescence intensity, are not comparable from experiment to experiment. Therefore we have opted to examine the ratios of the mean photosensitizer fluorescence values between Mac1$^+$ and Mac1$^-$ tumor cell populations. The contribution of low levels of "background red fluorescence" is further reduced by this ratio method, although it is not canceled completely, and the ratios may be slightly diminished.

The results for Mac1-stained cell suspensions obtained for FsaR and SCCVII tumors are shown in Fig. 3. The data are presented as ratios of the average $AlPcS_2$ or $AlPcS_4$ fluorescence in Mac1$^+$ *vs.* Mac1$^-$ cells, as explained in the figure caption. The ratios indicate higher levels of photosensitizers in Mac1$^+$ cells in both of these tumor models. The values for the denominator (average fluorescence intensity at greater than 625 nm in arbitrary units per cell) for $AlPcS_2$ were 0.97 ± 0.13 and 1.27 ± 0.28 and for $AlPcS_4$ were 1.48 ± 0.39 and 1.29 ± 0.32 ($\pm$standard deviation (SD)) for FsaR and SCCVII tumors respectively.

The preference for Mac1$^+$ cells is greater for $AlPcS_2$ than for $AlPcS_4$; the ratios obtained with $AlPcS_2$ were 1.49 and 1.75 for FsaR and SCCVII

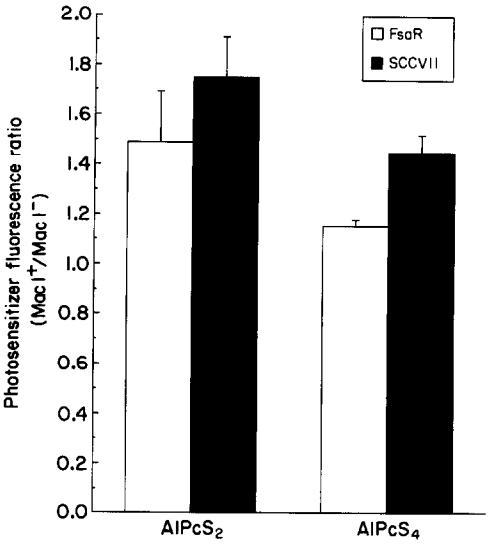

Fig. 3. Selectivity of $AlPcS_2$ and $AlPcS_4$ accumulation in Mac1$^+$ cells from FsaR and SCCVII tumors. Tumor-bearing mice were given $AlPcS_2$ or $AlPcS_4$ (10 mg kg^{-1}, i.v.) and sacrified 24 h later. The excised tumors were enzymatically digested and cells were stained for Mac1 antigen. In FACS analysis, photosensitizer fluorescence was recorded in Mac1$^+$ and Mac1$^-$ cells. The results are expressed as an average (from seven tumors) of the ratios obtained by dividing the mean fluorescence intensity of the Mac1$^+$ population by the mean fluorescence intensity of the Mac1$^-$ population. Bars are standard deviations.

tumors respectively. The ratios for $A1PcS_4$, however, were 1.16 for FsaR and 1.45 for SCCVII tumors. Statistical analysis showed that all the ratios were significantly greater than unity ($p < 0.005$), the ratios for $AlPcS_2$ were significantly higher than those for $AlPcS_4$ in respective tumors ($p < 0.01$ and $p < 0.025$ for FsaR and SCCVII tumors respectively) and the ratios for both photosensitizers were significantly higher in SCCVII than in FsaR tumors ($p < 0.05$ and $p < 0.005$ for $AlPcS_2$ and $AlPcS_4$ respectively). The average incidence of Mac1$^+$ population was $16\% \pm 3\%$ for FsaR tumors and $29\% \pm 7\%$ for SCCVII tumors. The observation that Mac1$^+$/Mac1$^-$ ratios for both photosensitizers were lower for FsaR than for SCCVII tumors may be a reflection of physiological differences (including perhaps phagocytosis activity) between TAM populations in these two tumors. Earlier work has indicated that the FcR$^+$/FcR$^-$ ratio for Photofrin also varies in different mouse tumor models [6, 7].

3.2. Hoechst 33342 plus FITC staining combinations

The DNA distribution profile of cells from FsaR tumors stained with Hoechst 33342 gives two clearly separated peaks, one diploid and the other aneuploid with a 1.7 times higher DNA content. This

enables the aneuploid tumor cells to be distinguished from tumor-infiltrating host cells (diploid) in flow cytometry analysis. Since Hoechst 33342 is excited by UV light and FITC by 488 nm light, the two-laser instrument allows cells to be stained and analyzed concomitantly with both of these dyes. Two such combinations, Hoechst 33342 plus FITC-IgG and Hoechst 33342 plus FITC-CD45 were examined in this work.

In these experiments both Hoechst 33342 and the photosensitizer are excited by the UV laser. When these two chromophores are excited with the same laser, there is some re-absorption of light energy from excited Hoechst 33342 by $AlPcS_2$ or $AlPcS_4$. This can be concluded from the higher fluorescence intensity observed in the red in the presence of Hoechst 33342 than in its absence. Light energy re-absorption results in the distortion of photosensitizer fluorescence values and this may be more pronounced in cells with higher Hoechst levels (*e.g.* aneuploid malignant cells). This also limits the validity of the fluorescence ratios, which can only be accepted as provisional. The ratios given below (based on the mean photosensitizer fluorescence values per cell for respective selected cell populations) should serve only for a general evaluation of the basic differences in photosensitizer content between the tumor cellular populations analyzed. These "provisional" ratios cannot be compared directly with $Mac1^+/Mac1^-$ ratios.

With the Hoechst 33342 plus IgG combination, four distinct cell populations can be selected and analyzed individually by the FACS. The malignant tumor cells are contained in the aneuploid FcR^- population (average incidence, 75%), and the photosensitizer fluorescence levels in the other three cell populations were related to the values obtained in this population, as shown in Table 1. The data for $AlPcS_2$ and $AlPcS_4$ fluorescence, obtained with individual tumors in two separate experiments, are listed in Table 1. The diploid FcR^- population should be composed predominantly of leukocytes with no detectable Fc receptor and with host non-immune cells also present. This population (average incidence, 13%) exhibited somewhat lower $AlPcS_2$ and $AlPcS_4$ fluorescence than the malignant tumor cells, but the significance of this difference is questionable in the light of limitations due to the light energy re-absorption with this staining combination. The diploid FcR^+ population (average incidence, 5%) showed markedly higher photosensitizer fluorescence (both $AlPcS_2$ and $AlPcS_4$) than the malignant cell population. As discussed earlier [6] and elsewhere [13], the cells in these mouse tumors that have a good expression of IgG-binding Fc receptor are almost exclusively TAM. Interestingly, the aneuploid FcR^+ population, with an average incidence of 7%, was also present. It showed considerably higher levels of photosensitizer fluorescence than the malignant tumor cells. The average size of these cells, according to forward light scatter in FACS analysis, was about 10% below the average cell size for the total tumor cell suspension. Since tumor cells, with the exception of some lymphomas, have no detectable Fc receptors, this aneuploid population should represent TAM with higher than diploid DNA content. In spite of the somewhat smaller size of these cells compared with malignant tumor cells, a possibility that they represent multinucleated macrophages known to be formed in the terminal stage of their development [14] cannot be ruled out. Alternatively, these cells could be either macrophages that have phagocytosed other cells or activated proliferating macrophages. This will be examined in future research.

The results for staining with Hoechst 33342 combined with FITC-conjugated monoclonal antibody to mouse CD45 (panleukocyte membrane marker) are shown in Table 2. Malignant tumor cells are contained in the aneuploid $CD45^-$ population (average incidence, 75%) and the results in the other three populations selected by this staining combination were compared with the photosensitizer fluorescence level obtained for this population. The diploid $CD45^-$ population (average incidence, 1%) represents non-immune host cells, and a vast majority of these should be endothelial cells. The levels of fluorescence of both photosensitizers in this population were not substantially different from that in malignant tumor cells. The diploid $CD45^+$ population (average incidence, 16%) should contain TAM and other tumor-infiltrating immune cells. The data indicate that the average $AlPcS_2$ and $AlPcS_4$ contents in these cells are markedly higher than in the malignant tumor cells. The existence of an aneuploid $CD45^+$ population (average incidence, 8%) again suggests the presence of immune cells with higher than diploid DNA content. This population, which also showed substantially elevated photosensitizer fluorescence, is presumably no different from the aneuploid FcR^+ population.

In contrast with the results obtained for Mac1 staining, the ratios of photosensitizer fluorescence obtained for diploid and aneuploid FcR^+ populations and for diploid and aneuploid $CD45^+$ populations do not indicate lower affinity for $AlPcS_4$ accumulation compared with $AlPcS_2$ in these cells.

TABLE 1. $AlPcS_2$ and $AlPcS_4$ fluorescence in FsaR tumor cell populations stained with Hoechst 33342 and FITC-IgG

	Tumor cell population			
	Diploid FcR^-	Diploid FcR^+	Aneuploid FcR^-	Aneuploid FcR^+
Experiment 1				
$AlPcS_2$ fluorescence[a]	0.15±0.01	0.53±0.04	0.19±0.01	0.71±0.05
Ratio[b]	0.80±0.11	2.75±0.30	1.00	3.68±0.27
$AlPcS_4$ fluorescence[a]	0.13±0.01	0.50±0.02	0.18±0.01	0.63±0.02
Ratio[b]	0.74±0.04	2.81±0.25	1.00	3.54±0.25
Experiment 2				
$AlPcS_2$ fluorescence[a]	0.37±0.04	1.34±0.08	0.47±0.04	1.47±0.15
Ratio[b]	0.80±0.10	2.87±0.43	1.00	3.13±0.21
$AlPcS_4$ fluorescence[a]	0.32±0.03	1.25±0.25	0.43±0.04	1.28±0.31
Ratio[b]	0.74±0.08	2.89±0.39	1.00	2.96±0.49
Average incidence (%)	13	5	75	7

[a]Average fluorescence intensity in the red (greater than 635 nm) in arbitrary units per cell. Note that these units are different in the two experiments. The photosensitizers (10 mg kg^{-1}, i.v.) were administered 24 h before the mice were sacrified and the tumor excised, enzymatically digested and stained for flow cytometry.
[b]Average fluorescence ratio, relative to the values obtained for the aneuploid FcR^- population (malignant tumor cells).
[c]The errors represent standard deviations.

TABLE 2. $AlPcS_2$ and $AlPcS_4$ fluorescence in FsaR tumor cell populations stained with Hoechst 33342 and CD45 antibody

	Tumor cell population			
	Diploid $CD45^-$	Diploid $CD45^+$	Aneuploid $CD45^-$	Aneuploid $CD45^+$
$AlPcS_2$ fluorescence[a]	0.14±0.02	0.58±0.02	0.15±0.01	0.52±0.02
Ratio[b]	0.94±0.09	3.74±0.22	1.00	3.41±0.32
$AlPcS_4$ fluorscence[a]	0.14±0.01	0.56±0.03	0.16±0.01	0.53±0.03
Ratio[b]	0.91±0.10	3.53±0.35	1.00	3.32±0.31
Average incidence (%)	1	16	75	8

[a]Average fluorescence intensity in the red (greater than 635 nm) in arbitrary units per cell. The photosensitizers were administered as described in Table 1.
[b]Average fluorescence ratio, relative to the values obtained with aneuploid $CD45^-$ population (malignant tumor cells).
[c]The errors represent standard deviations.

This apparent discrepancy may originate from limitations inherent in Hoechst plus FITC staining combinations, as discussed earlier.

Representative flow cytograms showing the distribution of $AlPcS_2$ fluorescence intensity in the four gated cell populations delineated by Hoechst plus FITC-CD45 staining are presented in Fig. 4. The data reveal considerable heterogeneity in the photosensitizer levels in the two populations containing TAM (diploid $CD45^+$ and aneuploid $CD45^+$) already suggested with $Mac1^+$ populations in Fig. 1. In contrast, malignant cell populations (aneuploid $CD45^-$) and non-immune host cells (diploid $CD45^-$) exhibit a more homogeneous distribution concentrated at low levels of photosensitizer fluorescence intensities. A similar pattern, *i.e.* a wide distribution of $AlPcS_2$ and $AlPcS_4$ levels with TAM populations and a narrower distribution concentrated at lower photosensitizer levels with malignant and host cells other than TAM, was seen with Hoechst plus FITC-IgG staining (not shown).

4. Comments and conclusions

Chan *et al.* [15] have used flow cytometry to sort cells from a mouse colorectal carcinoma into brightly and dimly fluorescing, based on the fluorescence of the photosensitizer chloroaluminum phthalocyanine (mixture of 1–4 sulfonates) that was administered earlier to tumor-bearing animals. These workers then characterized the cells in these two populations using morphological criteria. They

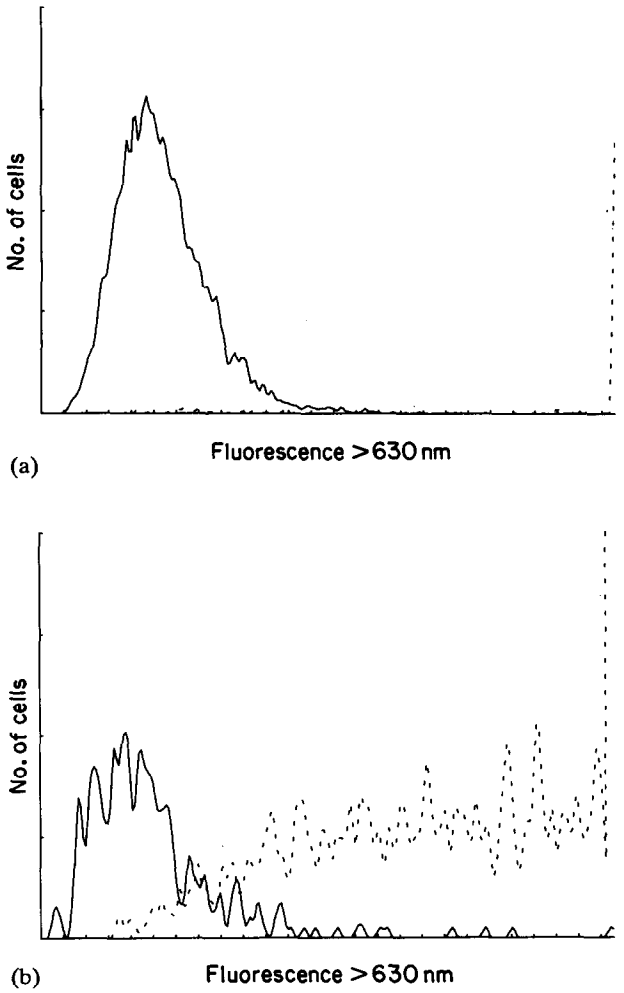

Fig. 4. Flow cytograms showing the distribution of $AlPcS_2$ in four cell populations from an FsaR tumor selected by combined staining with Hoechst 33342 and FITC-CD45. The photosensitizer was administered as in Fig. 1. (a) Aneuploid $CD45^-$ cells (full line) and aneuploid $CD45^+$ cells (broken line). (b) Diploid $CD45^-$ cells (full line) and diploid $CD45^+$ cells (broken line). The ordinate and abscissa are on a linear scale (see Fig. 1), but the ordinate scale in (b) is expanded 3.5 times compared with that in (a). There are 59% of aneuploid $CD45^+$ cells and 60% of diploid $CD45^+$ cells in channels 247–256 (including the overflow) at the right side of the ordinates.

found that 1 or 2 days after photosensitizer administration the majority of neoplastic cells and TAM were in the high fluorescing fraction, while the majority of lymphocytes and polymorphonuclear leukocytes were in the dimly fluorescing fraction.

The data presented in this work demonstrate that much more detailed analysis of photosensitizer distribution between different cell populations contained in solid tumors can be achieved by flow cytometry. Cell populations of interest can be distinguished by using monoclonal antibodies to specific membrane markers and this can be combined in some cases with fluorescent DNA staining. In previous work [6], we have shown that the assessment of photosensitizer content based on flow cytometry gives comparable results to those obtained using measurements based on the fluorometric assay from cell lysates.

The results of this work confirm that the TAM population is of dominant importance in photosensitizer distribution between different cellular populations contained in a solid tumor. We have been exploring ways to achieve optimal selection of the TAM population from cell suspensions obtained from enzymatically dissociated tumors. In addition to monoclonal antibody to mouse IgG employed in previous work [6], we have studied: combinations of staining for FcR or CD45 antigens, DNA staining, and staining for Mac1 antigen. We also wished to examine photosensitizer levels in tumor-infiltrating immune cells other than TAM and in endothelial cells from tumor vasculature. The representative population for endothelial cells should be the diploid $CD45^-$ population, while leukocytes, excluding TAM should be fairly represented in the diploid FcR^- population.

Flow cytometry analysis for $AlPcS_2$ and $AlPcS_4$ based on these staining combinations indicates that, with the exception of TAM, there are no substantial differences in photosensitizer levels in the other dominant cellular populations found in a solid tumor, including the neoplastic cells. It is further suggested that, although the average photosensitizer levels in TAM are higher than in the other tumor cell populations, these cells are markedly heterogeneous in photosensitizer accumulation. While some TAM have low photosensitizer levels, there is a fraction within this population characterized by extremely high levels of accumulated photosensitizer.

There are a number of factors which can account for this diversity in photosensitizer content within the same selected population. It can be assumed that individual macrophages are at different degrees of maturation and activation, which will affect their photosensitizer uptake [16]. In addition, small subpopulations of cells exhibiting different photosensitizer accumulation characteristics may be present in cases when the staining combination used does not select a completely uniform cell population. There may be a subpopulation of cells other than macrophages present in the $Mac1^+$ population (granulocytes), and other cells are present in the $Mac1^-$ population apart from malignant tumor cells (host immune and non-immune cells devoid of Mac1). Among the $Mac1^+$ cells there may also be monocytes that have infiltrated the

tumor after photosensitizer administration. Photosensitizer uptake into tumor cells may vary depending on the metabolic state of individual cells. The difference in cell size may also play a role; forward light scatter data recorded by FACS analysis for the complete tumor cell suspension indicates that the smallest 10% of the cells have approximately one-third of the cross-sectional area of the largest 10% of the cells within the whole population. Another factor which may be involved in this phenomenon is the distance of individual cells from blood vessels, with cells located closer to the vessel accumulating more photosensitizer than those cells which are more distant. This was confirmed in recent studies [17].

The photosensitizer $AlPcS_4$ is more hydrophilic than $AlPcS_2$, and it has been suggested that it has greater affinity for acellular rather than for cellular tumor structures [18]. Interestingly, no significant difference was seen in the levels of these two photosensitizers measured in the aliquots of tumor cell suspensions prepared for flow cytometry. The results, using fluorometric assay [19] of cell lysates from these aliquots, showed 9.2 ± 3.3 and 10.9 ± 4.9 ng of sensitizer per 10^6 cells for $AlPcS_2$ and $AlPcS_4$ respectively. A potential problem with the enzymatic digestion of tumor tissue is that it may include dye leaching which may vary with cell type and dye hydrophobicity. In related work with Photofrin [6], we have shown that some dye is lost by enzymatic treatment, but the loss in malignant cells and TAM is not significantly different. A similar control experiment with $AlPcS_2$ and $AlPcS_4$ indicated that cellular $AlPcS_4$ levels are not reduced by the enzymatic digestion procedure, while the detected loss of $AlPcS_2$ is somewhat lower than that seen with Photofrin.

Self-aggregation has been reported to be much more pronounced with $AlPcS_2$ than with $AlPcS_4$ [20]. This property has been assumed to determine the accumulation of photosensitizer in macrophages and other phagocytosing cells. However, we have found no substantial differences between $AlPcS_2$ and $AlPcS_4$ in the degree of preferential *in vitro* uptake by HL60ϕ cells (macrophages differentiated from human leukemia cells HL60) [21]. The results of this work with FsaR and SCCVII tumor models show that both $AlPcS_2$ and $AlPcS_4$ accumulate in TAM at higher levels than in other types of cells found in these tumors. However, $AlPcS_4$ is inferior in this respect to $AlPcS_2$. The selectivity of Photofrin accumulation in TAM appears to be greater than that of $AlPcS_2$ [6, 7]. This work suggests that even hydrophilic dyes, such as $AlPcS_4$, may exhibit a degree of preferential retention in TAM. It is also implicated that the direct phototoxic effect on TAM and other nonmalignant cells can be expected to be considerable, which should be a major element in the strong inflammatory reaction observed after PDT [22] and hemorrhagic necrosis leading to tumor destruction.

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