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Flow Cytofluorometric Analysis of Serial Biopsies of Tumours of the Uterine Cervix*

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Abstract—*The technique of flow cytofluorometry has been employed to assess, by means of cell suspensions prepared from serial biopsies, the radioresponsiveness of tumours of the uterine cervix. This enables DNA profiles and content of proliferating cells to be determined prior to treatment and during external beam and intracavitary therapy. Results show that elimination of hyperdiploid and hypertetraploid cells and reduction in the proliferating fraction of cells can readily be monitored by this method during therapy. This information, quickly available during treatment, may assist in estimating radioresponsiveness of the tumour and possible prognosis for the patient. Dose fractionation schedules may also be adjusted according to tumour response to therapy. Our results, however, show no relationship between histopathological classification of a tumour (WHO) and its ploidy state. The advanced stages of the disease (II and III) do, however, show an increased content of hypertetraploid cells in the tumour biopsies.*

INTRODUCTION

FOR SOME time means have been sought to determine the radioresponsiveness of carcinomas of the uterine cervix in the hope that these will reveal prognostic indices. In addition, potentially resistant tumours requiring other forms of therapy may be identified. A number of such investigations have employed histological examination of serial tumour biopsies [1] or vaginal smears [2], with a progressive decrease in malignant cells during therapy being taken to indicate a favourable prognosis [1, 2]. In some studies the response of tumours to half the planned dose of irradiation [1, 3] has been assessed whilst in others a test dose of irradiation followed by examination of cervical tissue was employed [4, 5]. However, these studies have relied on subjective assessments of morphological changes that may be difficult to interpret consistently. They also provide no information about the kinetic status of the tumours. With the introduction of flow cytofluorometry methods have become available for the rapid determination of DNA and RNA contents of individual tumour cells. This provides information that may be useful in assessing the potential radioresponsiveness of tumours and their kinetic status prior to and during therapy.

In our studies of uterine carcinomas we are applying flow cytofluorometric techniques to determine: (1) the ploidy status of tumours (including possible heterogeneity) and their proliferative indices; (2) the content of dying and dead cells; (3) the relationship of such parameters to the histological class and grade of tumour evaluated in parallel biopsies from the same tumour together with clinical stage; (4) the changes in these parameters during radiotherapy, and their relationship to radioresponsiveness of tumours; (5) with serial biopsies during radiotherapy the time at which tumour radioresponsiveness can best be established; and (6) the optimum dose fractionation schedule.

Tumour biopsies from over 100 patients have now been studied, the majority being followed over a full series of five fractional doses of intracavitary irradiation. In addition, serial biopsies at 1, 2, 3 and 7 days subsequent to irradiation together with a pretreatment biopsy have been examined from over 30 patients. In all cases histopathological assessment of parallel biopsies has been carried out. Patterns of particular interest with regard to radioresponsive-

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ness of cervical tumours are now beginning to emerge which we wish to report.

MATERIALS AND METHODS

Patients studied

Patients selected for assessment fell into two main categories:

(1) Those with clinical stage I disease who were treated with a radical course of combined intracavitary and external beam therapy (EBT). The intracavitary therapy and EBT were fractionated over 4 weeks into five intracavitary insertions each spaced a week apart and EBT on 4 separate days in between each intracavitary insertion. Intracavitary treatment was carried out using high-activity ^{60}Co sources and an afterloading (A Cathetron) as described by Joslin [6]. The dose delivered to the Manchester point A at each intracavitary treatment was 8.5 Gy and the dose to tissues in the region biopsied was approximately 2.5 times the point A dose.

(2) The other patients had late stage Ib, IIb or IIIb disease. These patients were initially treated with a fractionated course of EBT given on a daily basis. A total dose of 45 Gy was delivered in 20 fractions over 28 days. The dose per fraction delivered to the tissues being assessed was 2.25 Gy, in contrast to the approximate dose of 22 Gy per fraction delivered by the intracavitary therapy to the tissues being assessed.

Biopsies

Two pinch or punch biopsies were taken from immediately adjacent to the growing edge of the tumour or a single biopsy was taken and bisected. One was fixed in 10% buffered formalin for histological examination and the other was placed in phosphate-buffered saline (PBS) for flow cytometric examination. Tumours were biopsied immediately before start of intracavitary treatment, then weekly immediately before each intracavitary insertion. These times were varied somewhat for a few patients. Serial biopsies at 24-hr intervals were taken from certain patients to assess the early stages of cell response to irradiation. Those patients having EBT only had a biopsy immediately before treatment followed generally by serial biopsies at 1, 2, 3 and 7 days subsequent to irradiation and, where possible, subsequently weekly.

Tissue processing for flow cytofluorometry

Samples were processed within a maximum of 2 hr of the biopsy being taken. The tissue was minced using a scalpel, suspended in 4 ml HEPES (*N*-2-hydroxypiperizine-*N'*-2-ethanesulfonic acid) buffer, pH 7.3, with 1mM Ca^{2+} 200–800 units collagenase added (Type VII, Sigma Chemical Company, Poole, Dorset, U.K.) and the suspension incubated in a shaking water bath for 2 hr at 37°C. The collagenase aliquot was increased with increasing connective tissue content as assessed by tissue density. The disaggregated tissue and cells were collected by centrifugation, resuspended in PBS plus 2mM dithiothreitol then rotated overnight (16–18 hr, room temperature) in a rotary mixer. Cells were collected by centrifugation, then incubated with 1 unit of papain (Sigma Chemical Company) for 5 min at 37°C after resuspension in 2 ml PBS. Cells were collected and resuspended in 1 or 2 ml of PBS + 10% Hanks' basic salt solution (HBSS) and then an equal volume of PBS + 10% HBSS containing the stains was added to give a final concentration of 15 μM acridine orange (AO) and 3 μM ethidium bromide (EB). Cell numbers were adjusted so as not to exceed 1×10^6/2 ml final suspension. After 27 min staining the sample was placed in the flow cytometer and the sample flow started and allowed to equilibrate. After a further 3 min had elapsed data acquisition into computer memory was commenced. Data were transferred to computer disk immediately on completion of sample measurements. Samples of normal cervical cells, prepared from tissues removed at hysterectomy, were measured periodically for purposes of comparison.

Histopathology

The duplicate biopsies, or half of two or more bisected biopsies, for histopathological examination were processed by conventional methods, embedded in paraffin and one or more 5-μm sections from three levels stained by haemotoxylin and eosin. The sections were reviewed by two pathologists independently and graded using the WHO classification: large cell keratinising (LCK), large cell non-keratinising (LCNK) or small cell non-keratinising (SCNK) [7]. Adenocarcinomas have not been further subdivided due to the small number of cases. This grading system was chosen as it had been shown to be related to the radioresponsiveness and the subsequent behaviour of these tumours [8, 9].

Flow cytometric measurements

All flow cytofluorometric measurements were carried out on an Ortho Diagnostic Systems Cytofluorograf Systems 50H, with a Lexel 95-4W argon ion laser, routinely used at 250 mW at the 488-nm line. With the dichroic mirror and filter systems employed the wavelengths measured are green fluorescence (530–565 nm) and red fluorescence (>640 nm). With the staining method

used DNA content is proportional to green fluorescence intensity and RNA content to red fluorescence intensity [10]. Calibration of diploid (2c) fluorescence intensity was carried out with normal human lymphocytes stained in an identical manner to the tumour cells. Human lymphocytes were obtained from peripheral blood samples by layering onto lymphocyte separation medium (Flow Laboratories, Irvine, Ayrshire, U.K.). Only degenerating cells were permeable to EB under our staining conditions. The red fluorescence, with suppression of green fluorescence, resulting from entry of this stain was employed to separate the degenerating cells from viable cells on the flow cytofluorometric scattergrams (*vide infra*) [11].

Data storage and analysis

Data acquisition, storage, retrieval and analysis were carried out with an Ortho 2151 computer system. The computer programs available allow quantitation and statistical analysis of any cell subpopulation defined by means of a bit-map, within a scattergram on the computer graphics screen, in relation to the entire cell population of the tumour biopsy.

RESULTS

Initial results indicated that all pretreatment cervical tumour biopsies contained a considerable proportion of dying cells which, in subsequent biopsies from radioresponsive tumours, markedly increased 48–72 hr following radiotherapy. Control experiments showed this was not due to excessive time elapsing between the tumour biopsy and processing of tissues, or of the method of tissue disaggregation (*vide infra*), but that the presence of large numbers of dying cells is characteristic of all solid tumours. Such cells can seriously interfere with the analysis of fluorescence scattergrams as the staining intensity of DNA/RNA alters during degeneration [12]. Also of importance was the possibility that increase in content of dying cells during therapy may be helpful in indicating tumour response. We have, therefore, evolved a method employing supravital staining by AO, based on the differential permeability of viable and degenerating cells to the stain EB [11]. This separates the viable and dead and dying cells on the flow cytometric scattergrams and permits quantitation of the latter, with simultaneous measurement of ploidy and kinetic status of the viable cells (*vide supra*). Measurements to assess the effect of the tissue disaggregation procedure on the content of dead and dying cells, and the DNA distribution profiles, of the biopsies were carried out. Easily disaggregated tumour biopsies, initially prepared without use of enzymes, and cultured human lymphoid cell lines with 30–50% of the cells killed by glucocorticoid treatment, were employed. Comparative measurements following exposure to collagenase and/or papain under the routine conditions of time, temperature and enzyme concentrations showed that the numbers of dead and dying cells in treated and untreated aliquots were the same within an experimental variation of ±5%, with a comparable result for the DNA profiles. Measurements at intervals over an 18-hr period of gentle agitation in the presence of 2 mM DTT gave the same result. The success of the disaggregation method does depend on the purity of the collagenase used, and it is worthwhile testing various preparations to eliminate those with a considerable contamination of other proteolytic enzymes.

The basis for our method of analysis of the flow cytofluorometric scattergrams for the purposes of diagnosis, prognosis and assessment of response to therapy is depicted in Fig. 1. The scattergram is divided into 7 areas, and the percentage of cells in areas 4, 5 and 7 is determined. The percentage of cells in area 4 is calculated from: 100(number of cells in area 4)/(number of cells in areas 1–6). For area 5 the calculation is the same, with area 5 substituted for area 4. For area 7 the calculation is: 100(number of cells in area 7)/(total cells in scattergram). Percentage of cells for areas 4 and 5 is thus based on viable cells only. Area 1 is defined by the cell cluster obtained on a scattergram with normal human lymphocytes employing AO supravital staining. This corresponds to a diploid (2c) DNA content and a quiescent cell (G_0) RNA content. The lines at 2.5c and 4.5c are calibrated from the green fluorescence intensity, and the vertical line by the red fluorescence intensity, of the lymphocytes. Areas 2 and 3 are not used in the analysis. Area 4 includes all cells >4.5c (hypertetraploid cells). Area 5 includes all cells of 2.5–4.5c with cycling RNA content. This area will include the S and G_2 + M phases of diploid cells, malignant or non-malignant, plus abnormal cells in G_1 or early S phase that fall within this ploidy range. Area 6 includes G_1 diploid cells, plus polymorphonuclear leukocytes and macrophages that have infiltrated the tumour (characterised by increased red fluorescence on AO staining [13])(cf. area 6, Fig. 5E) and cells in the earliest stages of degeneration with membranes partially permeable to EB. A 'bridge' of dying cells [14] is often apparent passing through area 6 from area 5, especially following irradiation (cf. areas 5 and 6, Fig. 5F). Because of its heterogeneity we make no attempt to use this area for analysis. Area 7 includes dying cells with membranes permeable to EB.

Other workers have attempted to use the staining properties of cervical cells, in some instances combined with forward or right-angle light scattering, to give two-parameter discrimination between malignant and non-malignant cells [15–18]. We found that our staining procedure without prior fixation of the cells precluded this approach. Also, our principal objectives were to determine whether there is a relationship between tumour DNA profiles and patient prognosis, and whether changes in these profiles can indicate response of the tumour during therapy. As data were gathered, however, it became apparent that differences in DNA profiles were discernible between normal and malignant cervical tissue. We therefore included an attempt at diagnosis from the flow cytometric data in our objectives. We base our diagnosis, prognosis and estimate of response to irradiation on percentage content of cells in areas 4 and 5, and changes in these percentages, plus those in area 7, during treatment. We thus make no estimates of the distribution of cells within the cell cycle phases, these often being difficult, if not impossible, to determine due to aneuploidy/polyploidy of the tumours, plus abnormal accumulations of cells at certain points in the cycle (*vide infra*). This approach is based on measurements of normal cervical tissue (Fig. 2A) which have shown that the percentage of cells in area 5 does not exceed 20% and in area 4 does not exceed 2% for this tissue. In contrast, malignant cervical tissue shows values above 20% for area 5, with 90% of the tumours measured having values exceeding 30% (cf. Figs 2–5). These high values for area 5 in malignant tissue frequently appear to be due to abnormalities of synthesis in late S phase and inability to divide following G_2, leading to accumulations of cells which eventually die without completing division (Fig. 2B).

A comparison of diagnosis of malignancy based on this flow cytometric approach compared to histopathological evaluation is given in Table 1.

To determine if the DNA profiles determined from the pretreatment biopsies were related to histological classification the tumours analysed were divided into two groups: diploid tumours containing less than 2% cells of >4.5c (area 4) and hyperdiploid tumours containing greater than 2% cells of >4.5c (area 4). When these two groups were compared within each histological class (WHO) [7] a similar distribution was found for each class (Table 1), suggesting that DNA distribution profiles for cervical tumours are not related to histological classification. However, when DNA profiles were related to clinical stage a strong tendency was noted for the more advanced stages to be hyperdiploid.

Table 1. Comparison of flow cytofluorometric analysis and histopathological evaluation

	Malignant histopathology	Non-malignant histopathology
Malignant flow cytofluorometry*	100 (68.0%)†	15 (10.2%)†
Non-malignant flow cytofluorometry*	14 (9.5%)†	18 (12.2%)†

	Prognosis	
	Diploid‡	Hyperdiploid§
Histological class (WHO)		
LCK	11 (55.0%)‖	9 (45.0%)
LCNK	27 (57.5%)	20 (42.5%)
SCNK	17 (53.1%)	15 (46.9%)
Adenocarcinoma	9 (60.0%)	6 (40.0%)
Mean	(56.1%)	(43.9%)

*Assessed without knowledge of histopathological evaluation. Based on content of cells of 2.5–4.5c (area 5) exceeding 20% in pretreatment biopsy.
†Percentages of all patients assessed.
‡Diploid classification based on presence of less than 2% cells of >4.5c (area 4) in pretreatment biopsy.
§Hyperdiploid classification based on presence of more than 2% cells of >4.5c (area 4) in pretreatment biopsy.
‖Percentages within each histological classification.

The relevant values are presented in Fig. 3.

Three examples have been selected to illustrate the results of flow cytofluorometric analyses of serial biopsies taken during radiotherapy to assess the radioresponsiveness of the tumour. In Fig. 4 are depicted analyses of biopsies from a patient with stage Ib disease, taken pretreatment and at intervals during a course of intracavitary treatment. The elimination of cells of abnormal ploidy, followed by sterilisation of the irradiated field, may readily be discerned by examination of the percentages of cells in areas 4 and 5 of the scattergrams (Fig. 4). Area 7 of Fig. 4B also shows the considerable increase in dead and dying cells consequent upon the first intracavitary treatment; an early indication that the tumour is responding to radiotherapy.

In Fig. 5 are depicted analyses of a tumour biopsied during EBT of a patient with stage IIIb disease. This tumour showed little response until day 7 (Fig. 5D), but by day 9 few cells appeared in area 4 of the scattergram (Fig. 5E). The 'smear' of cells to the left in area 6 at 9 days (Fig. 5E) indicates infiltration by polymorphonuclear leukocytes and macrophages, which show increased red fluorescence due to some reaction of lysozomes with AO [13]. At 21 days (Fig. 5F) there is a considerable accumulation of radiation-damaged cells shown by the dense cluster of cells lying diagonally across the area 2/area 5

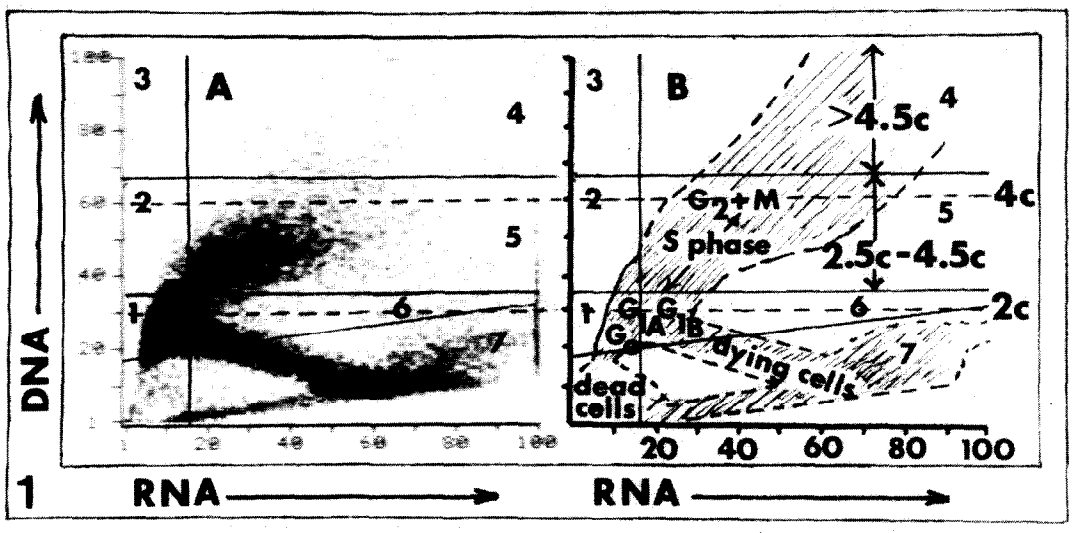

Fig. 1. Typical flow cytofluorometric scattergram of a cervical tumour with diagram to show division into 7 areas. Range of ploidy values within relevant areas, relation of areas to cell cycle phases (for diploid cells only) and dead and dying cells are shown. Areas 4, 5 and 7 provide a basis for diagnosis, prognosis and assessment of response to therapy, as decribed in Results. DNA content is proportional to green fluorescence intensity, and calibration of 2c and 4c is shown. RNA content is proportional to red fluorescence intensity for viable cells only (excluding area 7).

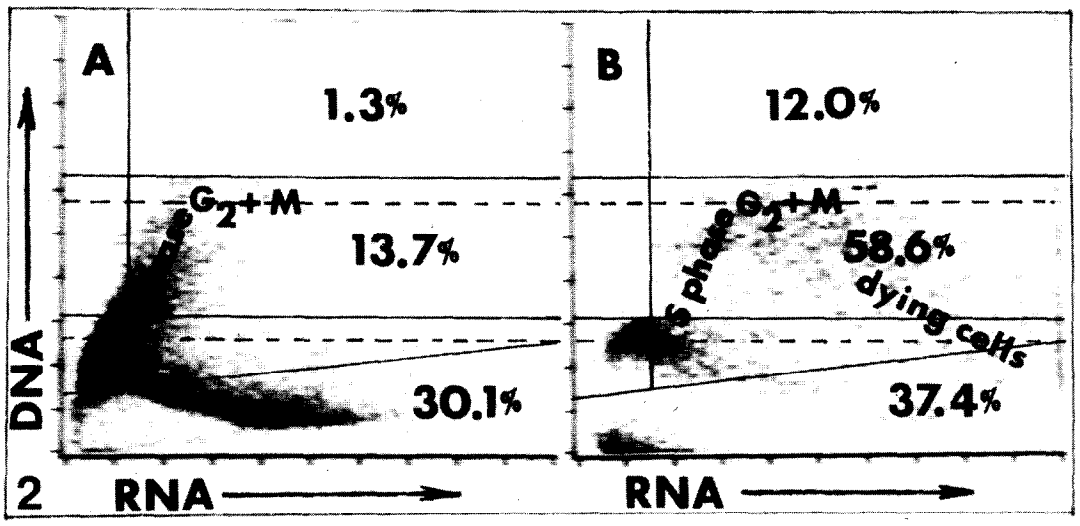

Fig. 2. (A) Scattergram of cells from normal cervical epithelial tissue. (B) Scattergram of cervical tumour cell suspension to show abnormal accumulation of cells in the late S and G_2 + M phases, with subsequent degeneration and death (area 7). In this and subsequent figures percentages of cells within areas 4, 5 and 7 are shown, calculated as described in Results.

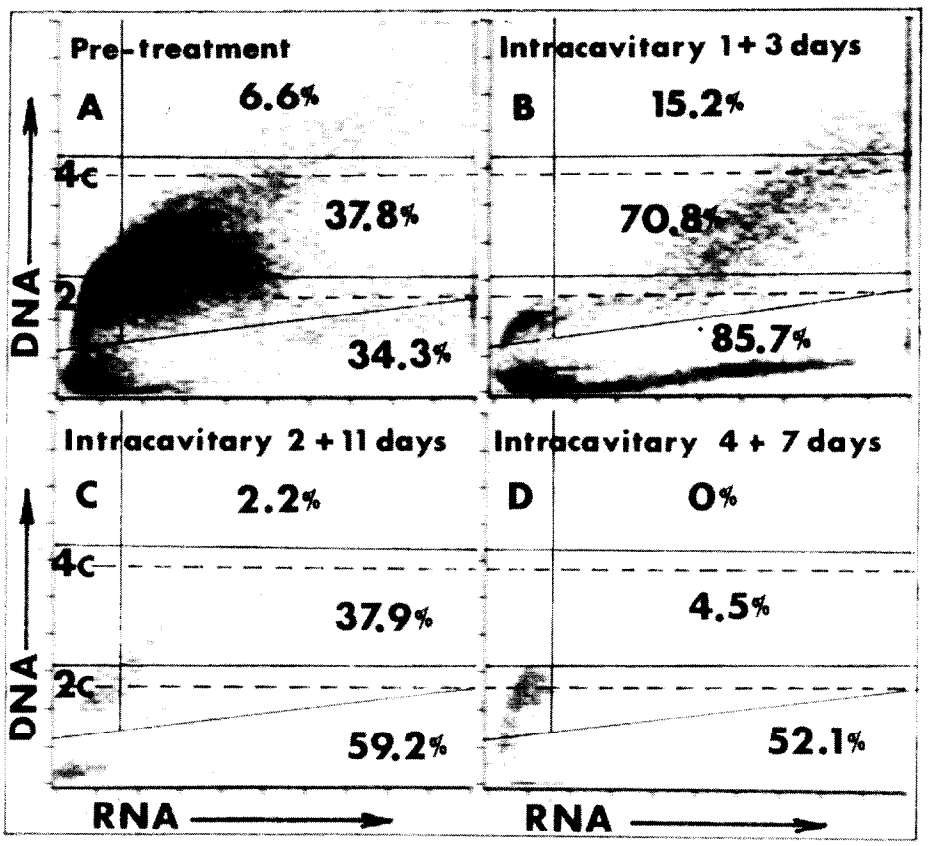

Fig. 4. Scattergrams of cell suspensions from serial biopsies of a cervical tumour during intracavitary therapy. The time during treatment at which each biopsy was taken is indicated in the figure. The division of the scattergrams into areas is described in the text. Patient stage Ib, large cell non-keratinising squamous carcinoma.

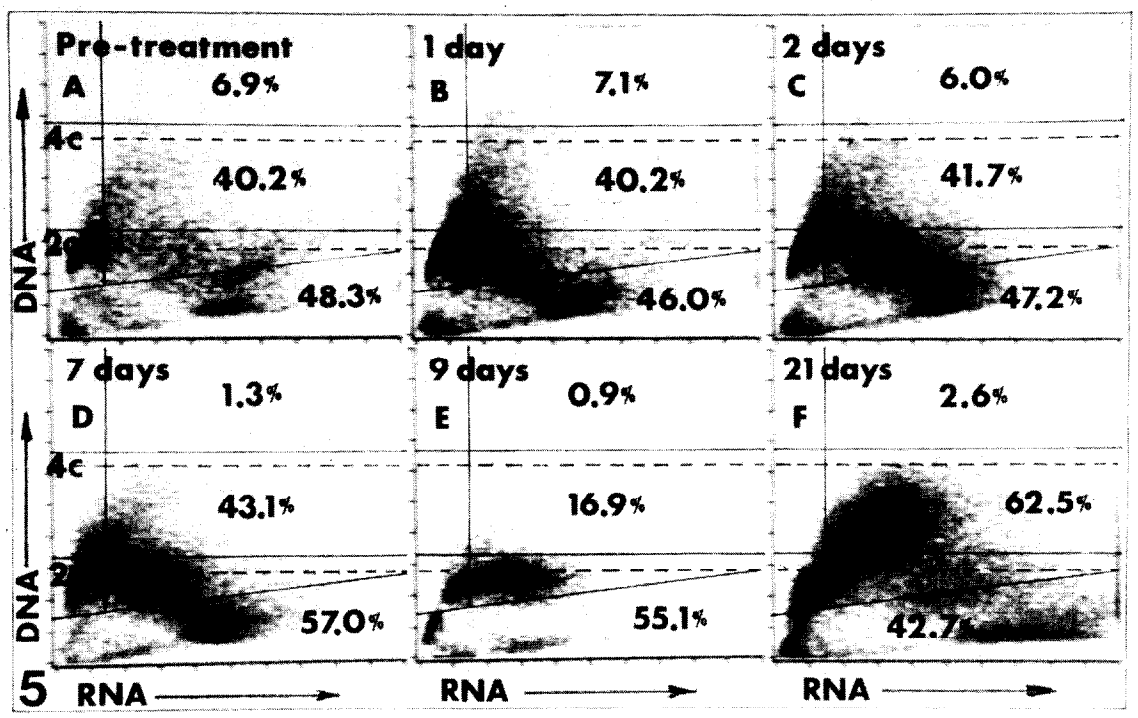

Fig. 5. Scattergrams of cell suspensions from serial biopsies of a cervical tumour during external beam therapy. Time of biopsy from start of treatment indicated in the figure. Patient stage IIIb, large cell keratinising squamous carcinoma.

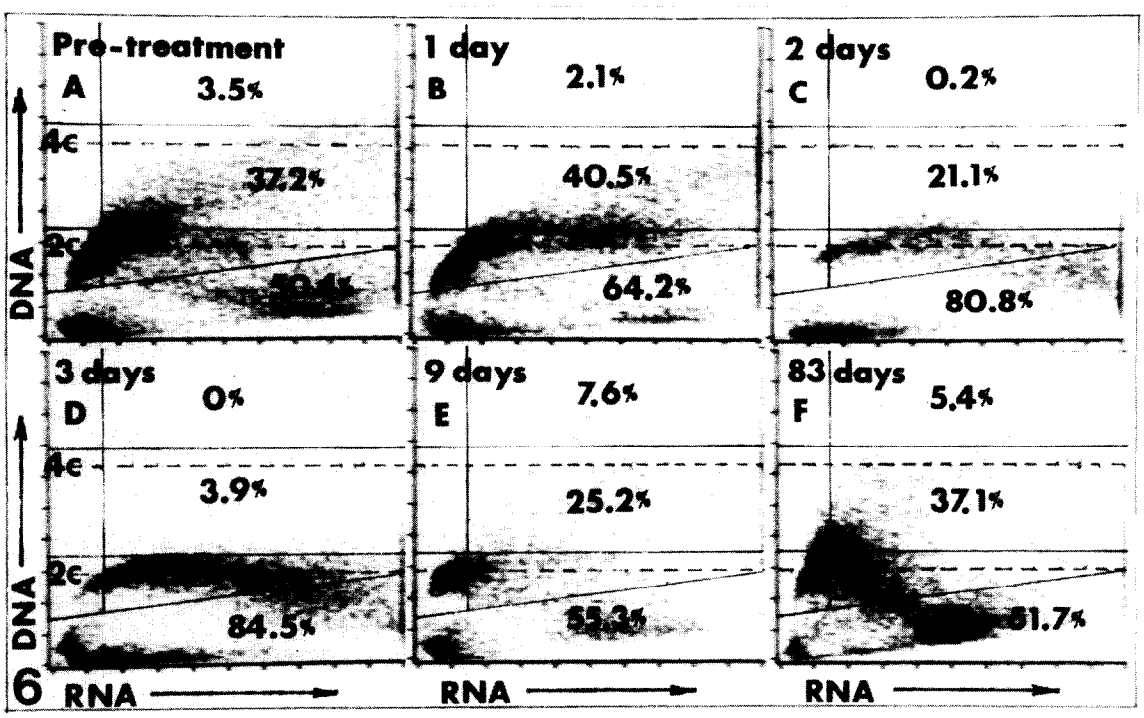

Fig. 6. Scattergrams of cell suspensions from serial biopsies of a cervical tumour during external beam therapy. Time of biopsy from start of treatment is indicated in the figure. Patient IIIb, large cell non-keratinising squamous carcinoma.

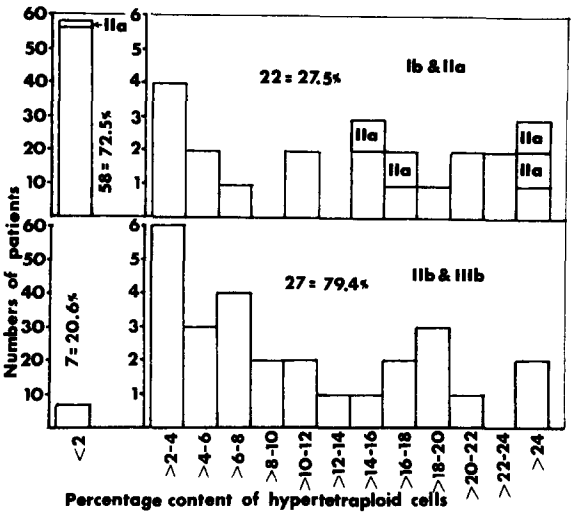

Fig. 3. Relationship between content of >4.5c cells (area 4) in pretreatment biopsy and clinical stage. (A) Stage IIa patients shown, remainder stage Ib. (B) Stage IIb and IIIb patients.

boundary. The 'bridge' of dying cells connecting this cell cluster to the dying cells in area 7 should be noted. The identification of these as radiation-damaged cells has been confirmed by scattergram patterns produced by the experimental irradiation of cultured lymphoid cells [Dyson, unpublished observations].

A further example of response to EBT is shown in Fig. 6. In contrast to the type of response seen in Fig. 5, this tumour showed an immediate and marked reponse to irradiation. Three days after start of treatment no cells appeared in area 4, and very few cells in area 5, of the scattergram, and there was a 34% increase in content of dying cells (cf. cell percentages, Figs 6A, B, C and D). By day 9, however, repopulation of the tumour was apparently underway, as shown by the reappearance of cells in area 4 (cf. Fig. 6E). Histopathological examination was in agreement with this finding and foci of tumour cells, apparently undamaged by irradiation, were evident within the biopsy. A biopsy taken immediately before the start of intracavitary therapy at 83 days (Fig. 6F) showed little alteration in DNA distribution profile as compared with the pretreatment biopsy (cf. Fig. 6A). Histopathological examination also confirmed the presence of viable tumour tissue in this biopsy.

DISCUSSION

The principal advantage of flow cytofluorometry is the speed with which it can measure the individual DNA and RNA contents of several thousand cells in suspension, thus presenting DNA and RNA distribution profiles for tumour biopsies within several minutes. This allows analysis of multiple biopsies to determine whether tumour heterogeneity may be present. It may also present a rapid means of assessing the response of a tumour to radiotherapy or chemotherapy, providing some property of the tumour that changes with response can be measured [19]. However, before such a technique can be widely applied for cancer diagnosis, prognosis or assessment of therapeutic response it is necessary to judge its value against other techniques commonly used for this purpose such as histopathology.

Diagnostic agreement between flow cytofluorometric analysis and histopathological evaluation is quite good (Table 1), and has improved as the project has proceeded. The major cause of disagreement initially was due to biopsy error and now, where possible, a single biopsy is taken and bisected, rather than two adjacent biopsies. Flow cytometry utilizes the whole of the tissue sample for the preparation of the cell suspension, in contrast to histopathology, which examines several levels through the biopsy. In some situations this may assist either method in detecting malignant cells. We appreciate that the criticism may be made that biopsy error, as above, or the presence of large amounts of stroma in the biopsy, could dilute the content of malignant cells in area 5, so reducing the value to the normal range (<20%). However, empirically the approach is working well, and with the last 33 patients complete agreement has been obtained between the two methods of evaluation. We would not suggest the method as suitable for cervical swab analysis, where few malignant cells may be present. Possibly automated image analysis may become more suitable for this, since it allows direct re-examination of cells of questionable classification [20], which flow cytometry does not. Some, however, have attempted to combine the advantages of both systems [17]. Results obtained by other workers also suggest that DNA distribution profiles, determined by flow cytofluorometry, may be of diagnostic and prognostic significance in cervical biopsy specimens [21].

Our results to date show no clear relationship between histological classification of cervical tumours and their ploidy status (Table 1). On the contrary, many well-differentiated tumours (LCK) contained large numbers of >4.5c cells, while many poorly differentiated tumours (SNCK) lacked such cells. The lack of a relationship is supported by analyses of other tumours, principally of the gastrointestinal tract, both by ourselves [22] and others [23]. However, a trend relating increased content of >4.5c cells to the more advanced stages of the disease (Fig. 3) is apparent. The number of patients is not yet adequate to establish a definite relationship, and tumours of some 27% of the stage Ib and IIa

patients have not conformed to this trend. Nonetheless, it is possible to speculate that this latter group represent more aggressive tumours, and a relationship may be found between the content of >4.5c cells in cervical tumours and the recurrence/survival rates. A definitive answer to this question awaits the outcome of patient follow-up over the next few years. A recent flow cytometric analysis of colonic carcinomas, however, showed that survival in a series of patients was directly related to the ploidy status of the tumour. After 60 months survival was 7.5% in patients with non-diploid tumours and 68% in those with diploid tumours [23]. Tumours affecting different tissues may, however, have different response patterns, so that extrapolation from one tumour site to another is inadvisable until the generality of such a relationship has been established [24].

The use of flow cytometry to establish radioresponsiveness of cervical tumours appears promising. While we normally carry out tissue disaggregation overnight, it can if necessary be completed within 2 hr, with the rapid availability of flow cytometric data. Thus examination of tumour response patterns may also allow individual modification of dose fractionation schedules to obtain a more favourable therapeutic ratio.

Our results suggest that caution must be exercised in relating the rapid elimination of cells in areas 4 and 5 to radioresponsiveness of the whole tumour during EBT. The same consideration may apply to intracavitary therapy, but here the much larger doses per fraction apparently do not allow the same partial recovery of the tumour to the extent observed with EBT. They also reduce the heterogeneity of response so that several distinct patterns of response to intracavitary therapy are beginning to emerge which promise to give an early indication of radioresponsiveness of the tumour. In contrast, during EBT many different response patterns have been observed which are, as yet, difficult to classify. For example, the serial biopsies depicted in Fig. 5 showed that 7 days elapsed before any appreciable response, as judged by elimination of cells from area 4, could be discerned (Fig. 5D). At 21 days large numbers of radiation-damaged cells were present and clinical examination showed the tumour to be regressing. The tumour subsequently responded well to intracavitary therapy. In contrast the serial biopsies depicted in Fig. 6 showed a very rapid response with no >4.5c cells present in the biopsy 3 days after starting EBT. However, by 9 days the tumour had apparently started to recover, and at 83 days from start of therapy the DNA distribution profile was essentially that of the pretreatment biopsy. The tumour did not respond to intracavitary therapy and histopathological evaluation confirmed the presence of viable tumour tissue on completion of therapy. The heterogeneous patterns of response observed during EBT suggest that if biopsies are only examined during the early stages of therapy misleading results could be obtained, due to the point in time at which tumour recovery may be detected (e.g. Fig. 6E) being omitted. Thus a test dose of irradiation as suggested by others [4, 5] could under certain circumstances be misleading. In contrast, when cervical tumours do not respond to intracavitary therapy this appears to become evident during the first 2–3 weeks of treatment. We have not, so far, observed tumour recovery during intracavitary therapy in the manner of the tumour depicted in Fig. 6.

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