Feulgen microdensitometry and analysis of S-phase cells in cervical tumour biopsies

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SUMMARY Tissue specimens from the cervical tumours of 70 patients undergoing radiotherapy were examined by Feulgen microdensitometry. Twenty-five of the 70 specimens were also subjected to in-vitro tritiated thymidine autoradiography to determine the proportion of DNA synthesising cells they contained. A spectrum of frequency distributions of nuclear DNA content was obtained from Feulgen microdensitometry, but by inspecting the data the basic DNA content of malignant cells could be established in most cases. Fifty-nine per cent of the tumours were ‘diploid’, 10% ‘tetraploid’, 13% ‘diploid to tetraploid’, and the remaining 18% ‘aneuploid’. Graphical analysis of DNA frequencies from 48 ‘diploid’ or ‘tetraploid’ tumours enabled the proportion of DNA synthesising (S) cells to be estimated by frequency distribution analysis. Estimates of the S component ranged from nil to 30%, were log normally distributed, and comparable to direct measurements of cells in S determined by autoradiography for 25 cases (range 1·2-28·7%). For all paired data the mean difference was 1·2 ± 1·45% (confidence limits), suggesting that overall Feulgen microdensitometry analysis may be an equally valid technique in providing cellular kinetic information with human tumour material.

The proportion of cells synthesising DNA (S cells) in malignant tumours of man may be estimated by tritiated thymidine (3H-TdR) autoradiography of biopsy specimens labelled in vitro (Fabrikant, 1971). With pretreatment material this method may be used to relate the proliferative activity of cervical tumours to clinical stage and to their response to radical radiotherapy (Tatra and Breitenecker, 1975; Dixon et al., 1977). Autoradiography, however, is not readily adaptable to routine clinical use.

In a proliferative population of normal diploid (2C) cells, each containing 46 chromosomes, the DNA content of individual nuclei will vary from 2C to 4C depending on position in the cell cycle as cells progress from G1 through S into G2. Thus it has been proposed that frequency distribution analysis of the nuclear DNA content of proliferative cells measured by Feulgen microdensitometry (Altman, 1975) or by flow cytofluorimetry (Crissman et al., 1975) may also be used to estimate the proportion of S-phase cells (Dean and Jett, 1974; Gray, 1974). If so, the method potentially offers a quick and reliable process for routine clinical laboratory assay.

For tumours, however, the method depends on the assumption that the malignant cell population is homogenous with respect to the basic (that is, G1) nuclear DNA content. Though an early Feulgen microdensitometric study of cervical tumours indicated that nearly all were near 2C or near 4C with respect of their modal G1 DNA content (Atkin et al., 1960), our own preliminary studies indicated that this was not so. Also the comparability of estimates of S-phase cells from Feulgen microdensitometry and flow cytofluorimetry by the more direct method of 3H-TdR autoradiography remains to be established for human tumour material.

Accordingly as part of an ongoing study of biopsies of tumour tissue from patients with cervical cancer we have routinely carried out microdensitometric measurement to determine the proportion of tumours which exhibit the characteristics of near 2C or near 4C through proliferative populations and therefore may be suitable for quantitative analysis of S-phase cells. With some biopsies we have also measured the proportion of cells in S by means of 3H-TdR autoradiography, thus enabling a comparison of the results of the two techniques to be made. The outcome of these studies is reported in this paper. The prognostic relevance of the microdensitometric information will be reported on after adequate long-term follow-up of the patients.

Received for publication 9 March 1977
Material and methods

Tissue for biopsy was obtained in all cases from the growing edge of the histologically confirmed tumours, presenting within the vagina but originating in the cervix, of patients immediately before undergoing radiotherapy. Approval of the study was obtained from the local medical ethics committee. The specimens were stored at 4°C in Hank’s balanced salt solution (BSS) for periods not exceeding two hours before being cleaned of adherent haemorrhagic and mucinous debris. They were then gently blotted dry and their surface (in some cases freshly recut) imprinted on clean, dry slides. These were then air dried, fixed in ether alcohol (1:1) for 30 minutes at room temperature, and rinsed in distilled water. Nuclear DNA in the imprinted cells was then demonstrated by the Feulgen reaction using the following schedule: hydrolysis for 45 minutes in 5N HCl at room temperature; two rinses in distilled water; Schiff’s reagent for one hour—basic fuchsin (CI 42510 RA Lambs, London) prepared by the method of de Tomasi (1936); two rinses in distilled water; three sulphite rinses of three minutes each. The slides were then washed in flowing tap water for 30 minutes, dehydrated, cleared in xylene, and mounted in DPX.

A Vickers M-85 Scanning Microdensitometer (Vickers Instruments Limited, York) fitted with a ×100 oil-immersion objective (Zeiss-Oberkochen) was used for measurement of the intensity of Feulgen staining. Integrated scans of individual intact nuclei were carried out at 545 nm without background subtraction. From each preparation 200 to 300 tumour nuclei were measured at random as they came into view along the axis of the field scan followed by manual adjustment of the microscope stage (Fig. 1). As a standard for evaluating the basic DNA content (‘ploidy’) of these tumour nuclei relative to that for non-dividing mononuclear cells 25-30 small lymphocyte nuclei, readily identifiable by their size and nearly always found to be present in these cervical tumour cell preparations, were also measured. Using the mean DNA value for these lymphocyte nuclei as a 2C standard, the frequency distribution of the tumour cells in the preparation was then plotted on a logarithmic scale to indicate tumour cell nuclei having a ploidy near to that of normal lymphocytes (2C) and higher modes of this amount (4C-8C, etc.)

In the larger biopsy samples, when 3H-TdR autoradiography could be readily carried out, the tissue remaining after imprinting for Feulgen staining was minced finely and then incubated at 37°C for 45 minutes in TC 199 supplemented with 15% fetal calf serum (Flow) plus 1 µCi/ml of 3H-TdR (Sp. Act. 5 Ci mmol⁻¹, Radiochemical Centre, Amersham) contained within a pressure capsule gassed with pure oxygen at three atmospheres absolute. The mince was then washed twice with BSS and then subjected to autoradiography and analysis according to methods detailed previously (Dixon et al., 1977). To avoid possible bias the analysis was carried out independently of the microdensitometric study using separate laboratory coding of the final slide preparations.

Results

Pretreatment biopsy specimens from a total of 70 patients have so far been subjected to Feulgen measurement and 25 of the 70 have in addition been analysed by 3H-TdR autoradiography of the same starting material.

Inspection of the frequency histograms obtained from biopsy imprints enables the ploidy and proliferative pattern of tumour cells to be established in most cases. The distributions from 48 biopsies were unimodal with major peaks at or near 2C and 4C (‘diploid’, Fig. 2a) or near 4C and 8C (‘tetraploid’, Fig. 2b), indicative in each case of a homogeneous or near homogeneous but proliferative clonal population. In nine cases measured DNA values ranged from near 2C to 8C with prominent modes at or near 2C, 4C, and 8C (Fig. 2c), suggestive of tumours containing a mixed population of proliferative diploid and tetraploid cells. In the remaining 13 specimens no clear ploidy could be ascertained and it may be inferred only that the tumours were composed of cells, proliferative or otherwise, with no characteristic basic DNA content.

Thus of the material subjected to microdensitometric measurement only about two-thirds (68%) exhibited a bimodal frequency distribution characteristic of a homogeneous though proliferative tumour cell population suitable for the analysis of S-phase cells. The method used for the latter purpose was a modified form of that first proposed by Mak (1965).

For a non-dividing (G0) population—for example, the lymphocytes found within cervical tumour preparations—the DNA distribution is unimodal (Fig. 3). When such data are replotted as a cumulative frequency on a probability scale against the logarithm of DNA content a straight line is obtained (Fig. 4a), the slope of which is a measure of the variance due to inherent experimental errors about the true mean of the measured population. A similar probit transformation of DNA frequency data for either a proliferative diploid or tetraploid tumour population (that is, as shown in Fig. 2a and b) produces a characteristically stepped curve (Fig. 4b). In these cases, however, the initial portion is attributable to
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the presence of cells with a $G_1$ nuclear DNA content and the final portion to cells with a $G_2$—that is, $2 \times G_1$—DNA content. Both these components of the curve when plotted as a function of the logarithm of DNA content would be expected to have the same slope since within a given population of cells the variance due to experimental method and machine error relative to the observed modal DNA content measured for $2C$, $4C$, or $8C$ cells is constant (for example, for liver nuclei (Dixon, unpublished)). On this basis the greater part of the intermediate portion of the cumulative frequency data with reduced slope may be attributed in a cycling population to the presence of cells in S. Also a quantitative estimate of this component may be obtained from the difference between the two intercepts formed by this part of the curve with those produced by extrapolation of the parallel lines drawn through the $G_1$ and $G_2$ components. For the example illustrated in Fig. 4b the estimate for the S-phase component is 12%.

Similar estimates of S-phase cells have been made for all 48 ‘diploid’ or ‘tetraploid’ tumours measured
by microdensitometry in the current series. The values so derived are log-normally distributed and may be compared with data obtained for 25 of the biopsies by means of 3H-TdR autoradiography (Fig. 5). A more detailed analysis of the paired data (Table) indicated that in six cases the difference may be regarded as significant (P < 0.05). For all cases, however, the mean difference (1.2 ± 1.45% confidence limits) was not significant (P > 0.05 t test for paired data)—that is, overall the two methods may be regarded as providing comparable values for the proportion of S-phase cells in cervical tumour biopsies.

Discussion

The observation that the measurement of nuclear DNA content by Feulgen microdensitometry produces a spectrum of frequency distributions with major 'ploidy' values for cervical tumours ranging from near 2C to 8C or more has been reported by Atkin et al. (1960). In their series of 124 cervical tumours, however, 45% were classified as near 2C and 53% near 4C with only 2% aneuploid. This contrasts with our series of 59%, 10%, and 18% for diploid, tetraploid, and aneuploid tumours respectively, the remaining 13% being clearly tumours with a mixed population of proliferative diploid and tetraploid cells (Fig. 2c). If ploidy is related to clinical spread of tumour (that is, stage) this difference may be a reflection of the relatively higher number of advanced cases so far included in our study.

Though cancer cells often have abnormal karyotypes (Atkin, 1971) most still exhibit clearly defined modal chromosomal numbers related to the modal DNA content measured by Feulgen microdensitometry (Atkin et al., 1966). Thus tumours which show DNA frequency distributions with only two peaks differing in their modes by a factor of two and also only contain cells of intermediate values (for example, Fig. 2a, b) may be considered homogeneous, or nearly so, in their basic G1 DNA content. Our current experience indicates that only about two-thirds of cervical tumours conform to these criteria and therefore may be considered suitable for frequency distribution analysis to determine the proportion of cells in S-phase of the cell cycle.
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Fig. 4 Cumulative frequency curves. (A) Lymphocyte data from Fig. 3. (B) Diploid tumour nuclei curves fitted free-hand by inspection of the data. Difference between arrows in curve B denotes that portion of the population attributable to cells in S phase.

Feulgen microdensitometry, however, would still represent a significant logistical gain over 3H-TdR autoradiography. The latter technique using conventional methods usually required four to eight weeks for isotopic exposure of the emulsion between labelling the specimen and carrying out microscopic analysis. This could be reduced to perhaps a few days by using high specific activity 3H-TdR (Livingston et al., 1974) or possibly even a few hours by rapid histological processing techniques and the incorporation of scintillator in the autoradiographic emulsion (Durie and Salmon, 1975). But the present microdensitometric technique is relatively simple and may be completed readily within 24 hours of receipt of the laboratory sample. Moreover, it may be further refined and automated by the existing technology of flow cytofluorimetry, provided cell suspensions may be prepared from small solid tumour biopsies—for example, by the use of ultrasonics (Dixon and Hustler, 1976).

Nevertheless, the further refinement of microspectrophotometric methods for clinical use in cancer treatment is justified only if the information derived reliably quantifies the proliferative cell population under examination and, moreover, if the information is of therapeutic relevance. With respect
Table  

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*Recurrent tumour in treated volume three months after radiotherapy.


We thank the Cancer Research Fund of Cookridge Hospital for the purchase of the Vickers M-85 Microdensitometer used in this study, and our clinical radiotherapy colleagues Professor C. A. F. Joslin and Dr A. J. Ward for providing material and for discussing the clinical aspects of this work.

References


Table  

*S Phase cells in tumour biopsies. Errors quoted (± 1 SD) based on total numbers of cells scored by means of each technique*
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doi: 10.1136/jcp.30.10.907

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