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DNA-Flow-Cytometric Measurements on the Normal, Atrophic, Hyperplastic and Neoplastic Human Endometrium*

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Summary. DNA distribution patterns and the fractions of the cell cycle phases were determined by means of flow-through cytometry in 87 samples of normal, atrophic, hyperplastic and carcinomatous human endometrium. The S-phase fractions vary during the normal menstrual cycle between 1 and 3% and reach a periovulatory maximum between 4.4 and 4.7%. Atrophic endometrium and regressive glandular cystic hyperplasia have little DNA synthesis (1.01% and 1.68% S-phase fractions respectively). Proliferating glandular cystic hyperplasia reveals 3.38% S-phase fraction, whereas adenomatous hyperplasia has an increased number of DNA-synthesizing cells (4.81%). The well-differentiated endometrial carcinoma shows no cytophotometrically detectable differences in comparison to adenomatous hyperplasia. All endometrial samples except for poorly differentiated endometrial carcinoma showed a diploid to tetraploid DNA distribution pattern. The poorly differentiated endometrial carcinoma displays two different types: one rapidly growing diploid-tetraploid tumor with 8.0 to 9.6% S-phase fractions, and another type with stemline deviations, polyploid nuclei and less pronounced synthetic activity.

Key words: DNA synthesis – Cell cycle – Flow cytometry – Endometrium

Introduction

The histological discrimination between various types of hyperplastic and neoplastic endometrial lesions may be extremly difficult in daily routine diagnosis of biopsy specimens. In particular it is difficult to establish whether

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the biological process is limited to a hyperplasia only, or whether an autonomous neoplastic differentiation has developed (Ferenczy 1979b). Endometrial samples containing such changes may be evaluated prognostically on the basis of the proliferative activity of the tissue samples, especially with regard to the number of DNA-synthesizing cells and the number of cells participating actively in the cell cycle.

Counting of mitosis on histological slides (Ahrens and Prinz 1957) is of little value for estimation of proliferative activity of tissue samples. Sources of error due to the method have been documented recently (Ellis et al. 1981).

Evaluation of the labeling index (LI) by means of autoradiography after incorporation of radioactive ^{3}H-thymidine (Fettig 1965; Nordquist 1970) has proved to be the most reliable scientific method for the estimation of the cellular growth rate. Unfortunately, autoradiography cannot be applied as a routine examination method in vivo.

Single cell cytophotometry on Feulgen-stained histological slides (Wagner et al. 1967, 1968; Sachs et al. 1974; Böhm and Sandritter 1975) is time-consuming. Moreover, it can be applied only to a small number of cells and the peaks are too broad.

On the other hand, automatic flow cytometry provides some fundamental advantages: the tissue or cell samples may be collected easily, and processing and staining of the samples is straight-foreward as well. A very large number of cells can be measured during a few minutes, the results are calculated automatically. Van Lindert et al. (1975) were the first to show that human endometrium can be evaluated by this method. Sprenger et al. (1978) have tried to incorporate flow cytometry into routine screening for endometrial carcinoma, with negative results.

Our own experience with this method in investigations of different human tissues and corresponding malignant neoplasms suggested that it could be useful for reassessing the applicability of flow cytometry to a large number of human endometrium samples. In particular we wished to establish whether changes of the cell cycle phases occur during the normal menstrual cycle, and whether endometrial hyperplasias may be delimited from each other as well as from normal and carcinomatous endometrium.

Materials and Methods

Altogether 87 endometrium samples were examined by means of automated flow-through cytometry. The samples were obtained from curettage and from hysterectomy specimens. Forty-two women had a well established history of a regular menstrual cycle. None of these patients had taken sexual hormones for 3 months prior the examination. Histological examination of the endometrium samples revealed normal endometrium of the proliferative or secretory phase corresponding to the day of the menstrual cycle. The cytometric results of this group were related to the menstrual cycle day, thus serving as reference values.

The remaining 45 samples derived from women with the following diagnoses: atrophic postmenopausal endometrium (12), glandular cystic hyperplasia (16), adenomatous hyperplasia (6) and adenocarcinoma (11). Histological slides from all these case were collected and evaluated by one of us (GEF), so that a uniform classification of the pathological cases could be achieved.

Small tissue samples were fixed immediately after removal from the uterus in 96% ethanol and afterwards sent to the Pathology Department for cytophotometric examination. The processing of the samples was carried out as described by Haag (1980): a representative part of the material was dried with blotting paper. The small tissue fragments were minced by scissors. After 10 min of shaking in a 0.25% pepsin solution, a suspension of nuclei was obtained. For DNA-specific staining of the nuclei, a solution containing ethidium bromide, ribonuclease and DAPI (4′-6-diamidino-2-phenylindole) was used. By appropriate dilution with fluorochrome solution, a cell density of about 10^5 cells per milliliter was obtained. Prior to recording flow cytometry histograms, the samples were passed through a 70 µm mesh.

The DNA-specific fluorescence was measured with an ICP 22 pulse cytophotometer (Phywe AG, Göttingen, FRG). Pulse height analysis was performed with a multichannel analyzer (MCA 8100, Canberra Ind., Meridan, CT, USA) displaying the distribution in 256 channels. The measurement data were transferred to a graphic computing system (GS 4051 combined with a digital plotter 4664, Tektronix Inc., Beaverton, OR, USA). First of all a logarithmic histogram was obtained on the screen. It was transformed after appropriate background correction into an arithmetic histogram which was printed out by the plotter. An integral curve of the corrected curve was also printed out. This curve served for the calculation of the different cell cycle phase fractions. The $\frac{G_2+M}{S}$ ratio and the proliferative index (PI) $\frac{(S+G_2+M)\times 100}{(G_{0/1}+S+G_2+M)}$ were calculated instantly. For further information on the measurement method and for the background correction procedure, see Haag (1980). Mathematical correction for clumped cell multiplets was performed after the formula given by Beck (1980).

Results

A) Normal Endometrium

The forty-two women with a stabile menstrual cycle who had received hormonal medication during the three months prior to cytometric examination showed normal premenopausal endometrium with menstrual cycle-concordant development in all cases. All the histograms of this group had a diploid stemline at 2C with a coefficient of variation (CV) below 5%. No case with polyploid DNA pattern occurred in this group.

The S-phase fractions as a percent of the total of cells measured per case (between 20 and 40×10^3) was correlated with the day of the menstrual cycle (Fig. 1a). The amount of DNA synthesizing cells varied between 1 and 2% during the first cycle days. It increases during the late proliferative phase and reaches a periovulatory maximum of 4.4 to 4.7% between cycle days 14 and 15. Afterwards the S-phase fractions decrease continuously to a lowest level of 1.25% between cycle days 20 and 21. During the late secretory phase, there is a second rise of DNA synthesis, with a maximum of 2.5 to 2.9% between cycle days 23 and 25. After cycle day 25 the S-phase fractions decrease again. The amount of G_2+M phases (Fig. 1b) attains nearly the same values as those of the S phases during the proliferative phase of the normal menstrual cycle. Therefore the ratio $(G_2+M):S$ was approximately 1 (Fig. 1c). This ratio changed in favour of G_2+M phases during the cycle periods of the secretory phase in which a decrease of DNA synthesis has taken place, i.e. between the cycle days 19 and 21 as well as between the days 26 and 28.

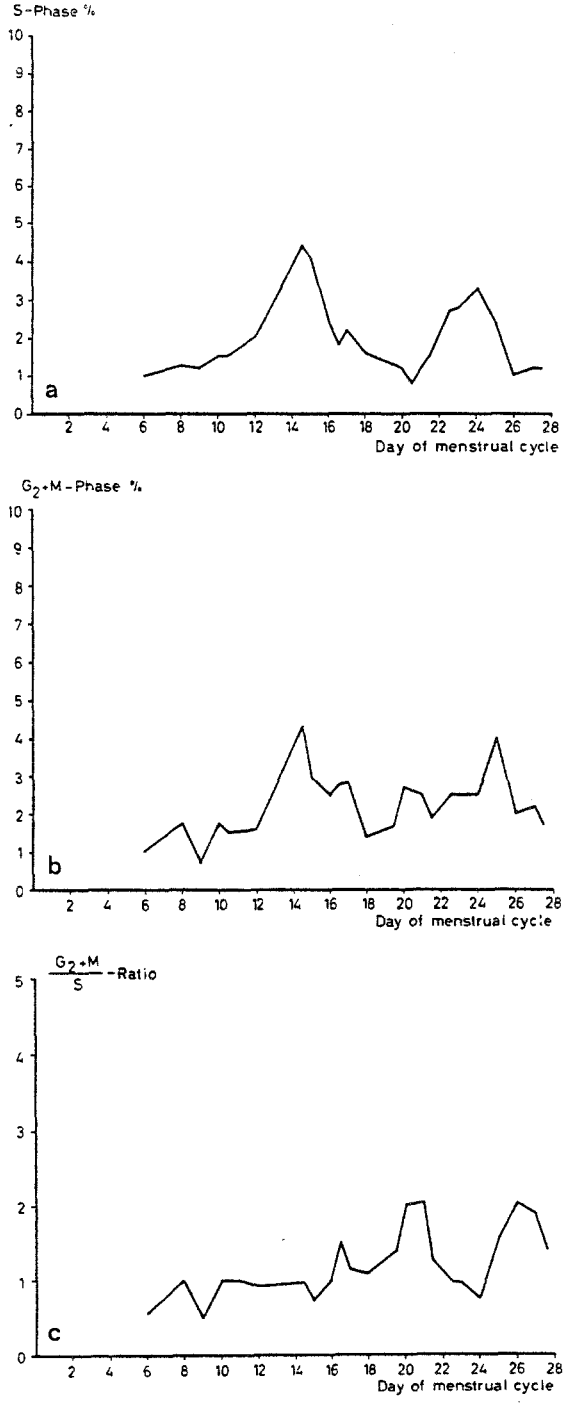


Fig. 1a–c. Cell cycle phases and (G_2+M):S ratio in the normal menstrual cycle. Considering the standard deviation significancy was found for the peaks in both **a** and **b**

Table 1. Results of flow-cytometric measurements of atrophic and hyperplastic endometrium and in well-differentiated adenocarcinoma

Diagnoses	*n*	S phase (%)	G_2+M phase (%)	PI	G_2+M:S
Atrophy	12	1.01 (0.59–1.43)	1.54 (0.02–3.06)	2.55 (0.73–4.37)	1.38 (0.32–2.44)
Glandular cystic hyperplasia (regressive)	7	1.68 (0.88–2.36)	1.84 (0.82–2.86)	3.52 (2.32–5.04)	1.10 (0.67–1.53)
Glandular cystic hyperplasia (proliferative)	9	3.38 (2.61–4.15)	2.53 (1.25–3.81)	5.91 (4.14–7.66)	0.75 (0.41–1.15)
Adenomatous hyperplasia	6	4.81 (4.50–5.12)	3.10 (2.24–3.96)	8.01 (6.91–9.11)	0.65 (0.49–0.81)
Adenocarcinoma (well differentiated)	5	4.76 (3.82–5.16)	3.20 (1.04–5.30)	7.96 (5.40–10.52)	0.66 (0.30–0.96)

B) Atrophic Endometrium

Endometrium samples of 12 postmenopausal women presented a histological picture of complete atrophy, without any signs of proliferative or secretory activity. The amount of DNA-synthesising cells varied between 0.4 and 1.6%, with a mean value of 0.7% (Table 1). Another 1.04% of the cells belonged to G_2+M phases, whereas the (G_2+M):S ratio reached 1.52 (Fig. 3).

C) Hyperplastic Endometrium

Altogether 22 endometrial samples corresponded to various types of hyperplastic changes of the endometrium. From 16 patients with the diagnosis of glandular cystic hyperplasia, seven cases had a fully developed regressive cystic atrophy with widely dilated cystic glandular ducts. In nine other cases, an actively proliferating glandular cystic hyperplasia was found. Adenomatous hyperplasia occurred in six patients. The following cytometric results were obtained in these cases (Table 1):

1. Glandular Cystic Hyperplasia. Regressive glandular cystic hyperplasia with a picture of a "Swiss cheese atrophy" do not differ significantly from atrophic endometrium from the point of flow cytometry. The amount of DNA-synthesising cells had an average value of 1.7% and thus exceeded the number of S phases in atrophic endometrium by only 1%. G_2+M phases were 1.84%, and the proliferating index (PI) 3.53. The (G_2+M):S ratio was 1.09.

Glandular cystic hyperplasia with proliferative activity displayed between 2.2 and 5.0% (medium 3.4%), and thus presented clearly more DNA synthetic activity than regressive glandular cystic hyperplasia. A simultaneous increase of G_2+M phases up to 3.81% (medium 2.53%) led to a PI of 5.92, and to a (G_2+M):S ratio of 0.75.

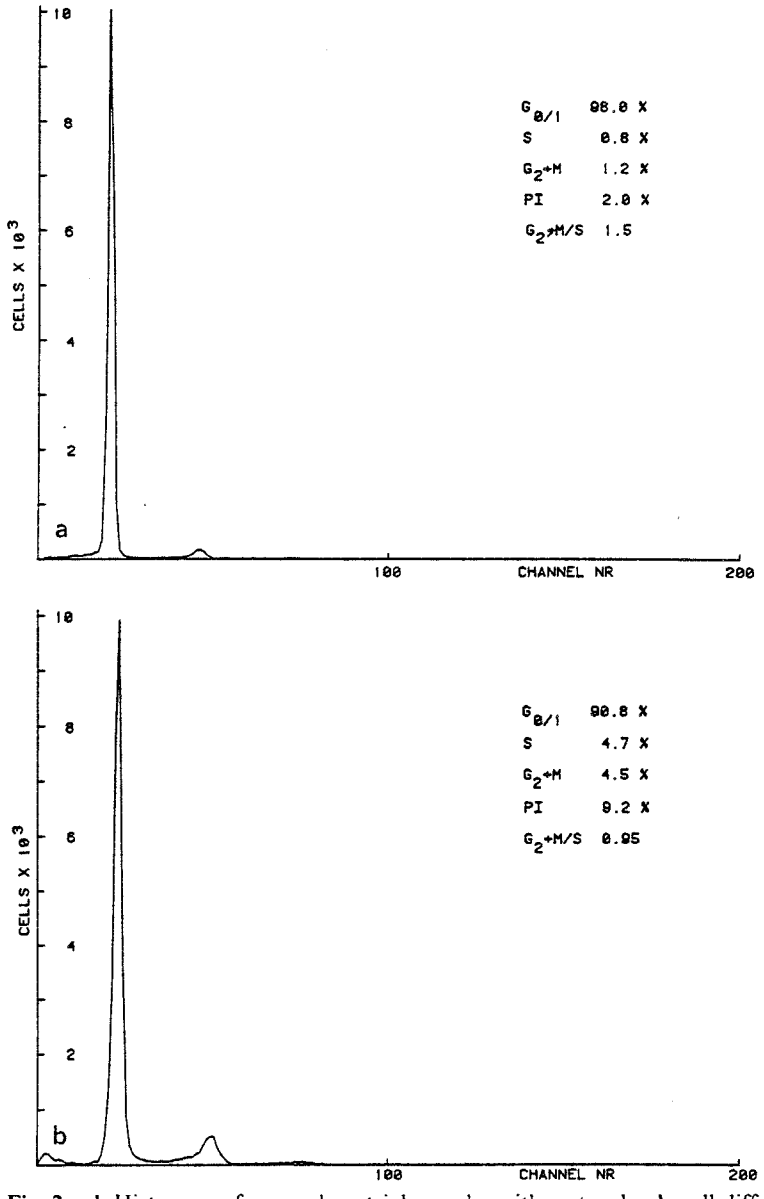
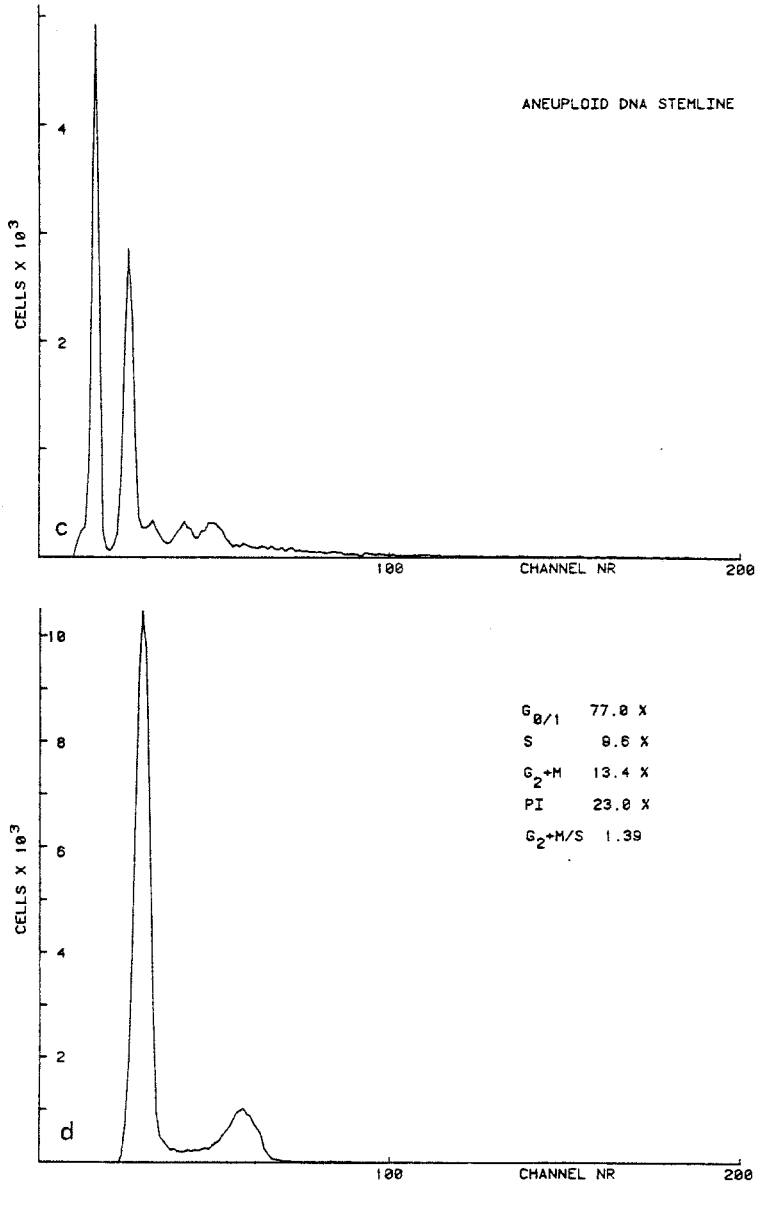


Fig. 2a–d. Histograms from endometrial samples with **a** atrophy, **b** well-differentiated carcinoma, **c** poorly differentiated carcinoma with a triploid stemline, **d** poorly differentiated carcinoma with a diploid stemline and a high DNA synthesis rate

2. Adenomatous Hyperplasia. In this group a further increase of S-phase fractions was registered (4.4–5.4%, medium 4.8%). The number of G_2+M phase fractions attained a medium value of 3.2% (2.24–3.96%), whereas the PI reached 8.0. The $(G_2+M):S$ ratio (0.66) presented a further decrease in comparison to the groups presented above.



D) Endometrial Carcinoma

We examined 11 endometrial carcinomas, six well differentiated and the other five were poorly differentiated. In the well-differentiated carcinomas the DNA-stemline was situated, without exception, in the diploid region. Neither stemline deviation nor polyploid DNA patterns could be observed in this group. The S-phase fractions varied between 4.0 and 6.5% (average

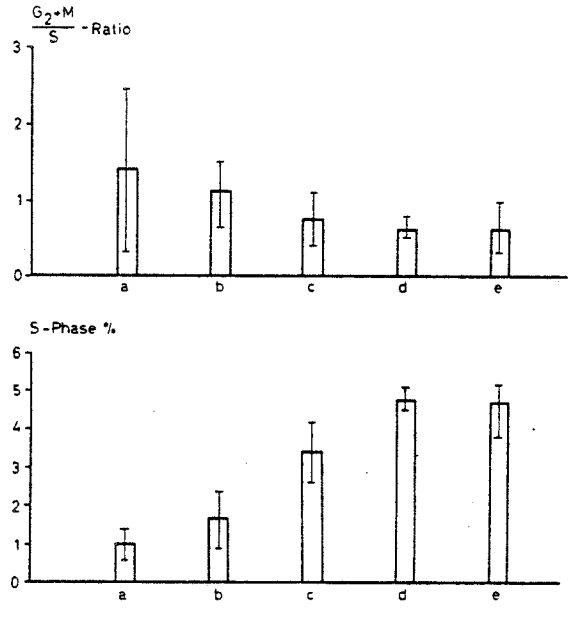


Fig. 3. S-phase fractions and $(G_2+M):S$ ratio in: *a* atrophic endometrium, *b* regressive glandular cystic hyperplasia, *c* proliferative glandular cystic hyperplasia, *d* adenomatous hyperplasia, *e* well-differentiated carcinoma

Table 2. Results of flow-cytometric measurements in 5 cases of poorly differentiated endometrial carcinoma

	S phase (%)	G_2+M phase	PI	$(G_2+M):S$	Aneuploid DNA patterns
a)	8.00	3.30	11.30	0.41	–
b)	9.60	13.40	23.00	1.39	–
c)	2.46	1.20	3.66	0.55	Tetraploid stemline Polyploid nuclei in C 8
d)	4.20	11.20	15.40	2.66	Polyploid nuclei in C 8
e)	Not calculable because of superposition of tumor cells with the G_2+M phase of the diploid cell line				Triploid stemline

4.76%). The proliferative index (PI) was 7.96, and the $(G_2+M):S$ ratio calculation gave a value of 0.67.

In contrast to well differentiated carcinoma, the poorly differentiated carcinomas of the endometrium reveal more evident differences in comparison with normal endometrium: among five malignancies in this group, only two had diploid and tetraploid DNA patterns (Fig. 2c) but with a marked rise of S-phase fractions to 8.0 and 9.6%, and, in addition, an increase of the PI up to 11.3 and 23.0 respectively (Table 2). The remaining three undifferentiated carcinomas showed aneuploid DNA patterns: one case had a triploid DNA-stemline with a hexaploid duplication peak (Fig. 2d). The G_2+M phase of soma cells superposed with the S-phase fractions of the tumour cells, so that the number of DNA-synthesizing tumor cells could

not be evaluated. Another case showed a tetraploid DNA stemline with 8C polyploidies, but with a surprisingly low number of S-phase fractions (2.4%). The calculated PI in this case was only 3.66; the $(G_2+M):S$ ratio was 0.50. The third poorly differentiated endometrial carcinoma had 4.2% S-phase fractions, 11.2% G_2+M phases and polyploid nuclei at the 8C level. The proliferative index was 15.40 and the $(G_2+M):S$ ratio was 2.66.

Discussion

Our studies on the human endometrium have revealed that human endometrium samples may be examined by means of automated DNA flow-trough cytometry without appreciable technical problems. When the examination procedure is carried out correctly, histograms which can be readily evaluated are obtained. The method thus meets high standards.

We were able to demonstrate for the first time using this method that the cell cycle phase fractions are related to the days of the normal menstrual cycle. The course of S-phase fractions in the normal endometrium coincides well with the physiological variations of serum oestrogen levels. The proportion of DNA-synthesizing cells varies between 1 and 3%. This limit is exceeded only during the periovulatory phase (day 14 and 15), during which the intensity of DNA synthesis is comparable with that of hyperplastic and neoplastic endometrium. Comparing our results with literature data on DNA synthesis during the normal menstrual cycle, we found that our data are rather close to those reported by Nordquist (1970). Nordquist measured the LI after in vitro incorporation of radioactive labeled ^{3}H-thymidine in samples of normal human endometrium. He found menstrual cycle-dependent variations of the LI, which were similar to the number of the S-phase cells in our series. Solely the S-phase fractions of the periovulatory peak are somewhat higher in our series (4.4–4.7%) than the LI reported by Nordquist for the same menstrual cycle phase (1.75–3.25).

As reported by Van Lindert et al. (1975) after examinations of normal endometrium by means of flow cytometry, the S-phase fractions were much higher during the late proliferative phase (8–10%) than in our series. This marked difference may be explained by the fact that Van Lindert et al. (1975) did not apply background correction procedures to their histograms which explains the higher S phase fractions in their series.

Whereas it is not difficult to understand the increase of DNA synthesis from the beginning of the menstrual cycle up to the periovulatory period as a consequence of increasing oestrogen levels and their influence on the glandular epithelia, interpretation of the second S-phase peak during the secretory phase proved to be less simple. Automated cytometry does not allow differentiation between glandular epithelia and stromal cells. Since the investigations of Fettig (1965) the increase of synthetic activity during the second half of the menstrual cycle is considered to be due to an exclusive activation of the stromal cells (Norquist 1970; Ferenczy 1979a). We cannot comment on this question at the present state of our study.

Hyperplastic Endometrium

Fully developed glandular cystic hyperplasia shows a synthetic activity resembling that found during the oestrogen phase of the normal menstrual cycle. The stemlines do only appear in the diploid sector with a tetraploid reduplication peak. However, the amount of DNA-synthezising cells do achieve values up to 5% in some isolated cases, which represents a considerable proliferative activity for the human endometrium. Regressive glandular cystic hyperplasia with cystic dilated glandular ducts is characterized cytophotometrically by a low proliferative activity (1.68% S-phase fractions), similar to that of postmenopausal atrophic endometrium.

Automated flow cytometry might be a useful method for prognostic prediction of the further development of the hyperplastic change in the case of glandular cystic hyperplasia and for followup control examinations.

With a median value of 4.8% S-phase fractions adenomatous hyperplasia shows more DNA synthesis than does glandular cystic hyperplasia. The DNA pattern is in this group also pure diploid with a tetraploid peak of the $G_2 + M$ phases.

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The figures in this group were clearly higher than in normal endometrium, except the periovulatory phase. Assuming that the menstrual cycle history is well known and no hormonal therapy was carried out on the patient, it should be possible to delimit adenomatous hyperplasia from normal endometrium and from most cases of glandular cystic hyperplasia using the number of S-phase fractions and the $(G_2 + M):S$ ratio.

Endometrial Carcinoma

The histograms of well-differentiated and of poorly differentiated endometrial carcinoma clearly differed. Whereas the well differentiated carcinoma showed neither stemline deviations or polyploid peaks, the poorly differentiated carcinomas displayed such changes in 3 of 5 cases. When only diploid cell lines occurred in poorly differentiated carcinomas, a marked increase

of the S-phase fractions of 8.0–9.6% could be observed. The DNA synthesis was much higher than in well differentiated carcinomas. This finding is of a certain importance. The extent of the number of S-phase fractions prove to be another criterion of malignancy, apart from stemline deviation and polyploidisation.

Delimitation of well-differentiated carcinoma by means of automated cytometry is not as easy as in the poorly differentiated carcinomas. The number of S-phase fractions does not differ from that of adenomatous hyperplasia. The PI is 9.2% in both groups. Similar results on the DNA content and on the proliferative activity of endometrial carcinoma were repeatedly reported in the literature: Wagner et al. (1967) found a diploid to tetraploid DNA distribution in all 6 cases of glandular cystic hyperplasia and in eight out of ten cases of adenomatous hyperplasia. The remaining 2 cases of adenomatous hyperplasia as well as six adenocarcinomas (three well-differentiated and three poorly differentiated) revealed aneuploid DNA patterns. It should be mentioned, however, that these examinations were carried out on 50–100 nuclei per case only, and that errors due to the method cannot be excluded completely. Sachs (1974) found diploid and tetraploid DNA values in well differentiated carcinomas of the endometrium, and he was able to report the finding of a tetraploid DNA stemline in the case of a poorly differentiated carcinoma. Böhm and Sandritter (1975) found a diploid stemline with a tetraploid duplication peak in a well-differentiated carcinoma as well as in one case each of a metastasizing papillary carcinoma, of an undifferentiated carcinoma and of a carcinosarcoma, they found hypertetraploid and polyploid DNA values.

Sprenger et al. (1978) could hardly differentiate between malignant and nonmalignant endometrial changes on the basis of cytometry alone. On the other hand our examinations on human endometrium with automated flow-through cytometry led to a more favourable result than the study of Sprenger et al. (1978). This may be the result of different technical conditions in the two studies. The Sprenger group examined exfoliated cells and superficial endometrial cells collected by means of the Jet-wash method. The cells obtained in this way are often in a state of necrobiosis which may exercise a strong influence upon the staining process. Accordingly, the histograms of the Jet-wash specimens revealed broad peaks and a high CV. Consequently their evaluation was rendered more difficult and limited the overall information provided by Sprenger's study.

Conclusions

1. Human endometrium may be examined by means of automated flow-through cytometry without appreciable technical problems.

2. Normal endometrium shows a diploid stemline with a tetraploid duplication peak. There are no polyploid nuclei. The S-phase fractions are closely related to the day of the normal menstrual cycle. The course of S-phase fractions coincides well with the physiological variations of serum oestrogen levels.

3. Atrophic endometrium, regressive and proliferative glandular cystic hyperplasia and adenomatous hyperplasia also present diploid DNA stemlines only with a tetraploid duplication peak. The S-phase fractions increase in proportion to the degree of hyperplastic change. At the same time, the $(G_2+M):S$ ratios decrease continually in the sequence from atrophy to adenomatous hyperplasia.

4. The flow cytometric pattern of the well-differentiated endometrial carcinoma reveals a pure diploid-tetraploid DNA distribution. Neither stemline deviations nor polyploid DNA values are found in this tumour. Thus there are no cytophotometrically detectable differences between adenomatous hyperplasia and well differentiated adenocarcinoma of the endometrium.

5. The poorly differentiated carcinoma differs cytophotometrically from the well-differentiated carcinoma in the following aspects:

a) an increased S-phase fraction up to about 8–9%, combined with a pure diploid-tetraploid DNA distribution, and

b) stemline deviations or the appearance of polyploid nuclei in combination with simultaneous decrease of S-phase fractions.

6. Assuming that the menstrual cycle history is well known and no medication containing sexual hormones was given to the patient, it should be possible to delimit adenomatous hyperplasia and well differentiated carcinoma from other endometrial changes. The poorly differentiated endometrial carcinoma can be recognized more easily on the basis of the criteria mentioned above.

7. Under these circumstances, automated cytometry can be used as an additional method in endometrial diagnosis, especially in routine screening for hyperplastic and malignant changes of the endometrium. The final diagnosis depends on histological study.

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