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AN APPROACH TO THE PROBLEM OF HETEROGENEITY OF HUMAN TUMOUR-CELL POPULATIONS

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Summary.—1. Successive sampling of ovarian cancers during cytostatic treatment showed several cases of notable changes in their ploidy distribution and one change in modal chromosome number, indicating selection of a resistant tumour-cell population. 2. Studies of cell suspensions from human tumour specimens incubated with [^{3}H]-TdR after exposure *in vitro* to various cytostatic agents have shown variation in labelling between different parts of the same tumour, as well as between the primary tumour and its metastases or ascitic tumour-cell population, which may be accounted for by variation in sensitivity of the tumour-cell population. 3. Studies of nuclear morphology in 20 endometrial cancers before and after progesterone therapy demonstrate considerable variation in the proportion of cells undergoing secretory conversion within the same tumour, indicating primary heterogeneity of the tumour-cell population in response to progesterone.

PERSISTENCE and early recurrence of human tumours after cytostatic or hormonal treatment starts from surviving cells. Such cells may be less sensitive due to natural primary resistance to the treatment. This postulates that the tumour-cell population is heterogeneous for sensitivity to various cytostatic drugs or hormones.

Cytogenetic and cytochemical studies have demonstrated that some experimental as well as human tumours are composed of more than one cell clone, which often differ significantly in their biological characteristics (Bishun *et al.*, 1973; Mian *et al.*, 1974; Mitelman, 1971; Palyi *et al.*, 1977). Several studies indicate that this biological heterogeneity of the tumour-cell population is also expressed in different sensitivities to various cytostatic drugs, both in animal and human tumour-cell lines (Hakansson & Tropé, 1974; Tropé & Hakansson, 1974; Tropé *et al.*, 1975; Whisson & Siracký, 1969).

There is still the problem of estimating or characterizing the type and degree of cellular heterogeneity of human tumours in respect of their sensitivity to various therapeutic agents, at the cellular level and *in vivo*.

In the present investigations we have attempted clinical interpretation of the problem of cellular heterogeneity in different types and sites of human tumours.

MATERIALS AND METHODS

Ploidy in ovarian cancers during cytostatic treatment.—The study comprised 12 inoperable ovarian cancers with primary chemotherapy (Leukeran, Burroughs Wellcome, London; Mannogranol, Gedeon Richter, Budapest; TS-160, SPOFA, Prague; Cytembena, SPOFA, Prague). Before, during and 2–3 days after terminating the cytostatic treatment, repeated chromosomal analyses (at about 10-day intervals) of cancer cells from ascitic fluid were performed. The first samples were taken immediately before cytostatic treatment.

Within a few minutes of withdrawal, Colcemid (CIBA, Basel; 2 mg/l) was added to the ascitic fluid for 2 h at 37°C. Cells were then treated with hypotonic 1% sodium citrate solution for 30 min and fixed repeatedly in methanol–acetic acid (1:3). Air-dried slides

were stained with Giemsa solution, metaphases photographed, the chromosomes counted and summarising histograms produced.

Incorporation of ^{3}H-TdR in tumour-cell suspensions after 3 h exposure in vitro *to various cytostatics.*—If human tumours are heterogeneous in their sensitivity to cytostatic drugs, the sensitivity results from small biopsies in an *in vitro* test are not likely to be representative of the whole tumour. To overcome this problem the following studies were made:

1. Comparison of different regions, as distant as possible, of the same tumour (one ovarian and 2 colon cancers).

2. Comparison of primary tumours with their metastases and/or comparison of different metastatic nodes of the same patient (one ovarian cancer, primary tumour and omentum metastasis; and one ovarian cancer, different metastatic nodes only).

3. Comparison of primary tumours and their ascitic tumour-cell populations (one ovarian and one gastric cancer).

All specimens were obtained at operation. The tumour material was brought into cell suspension. The cells were washed in MEM, resuspended, assayed for cell viability by Trypan blue, and distributed into test tubes (10^7 viable cells in each). The tumour cells were then incubated in MEM with cytostatic drugs for 3 h (Alkeran (ALK), Burroughs Wellcome, London; Ftorafur (FF), Medex, Moscow; Fluorouracil (FU), Hoffmann-La-Roche, Basel; Oxauracyl (OXAUR), VUFB, Prague; Methotrexate (MTX), Lederle, Pearl River, N.Y.; TS-160, SPOFA, Prague; Vinblastine (VINBL), Gedeon Richter, Budapest; Vincristine (VINCR), Gedeon Richter, Budapest). Drug concentrations corresponded to those used for treatment in man (mg/g body wt converted to mg/ml medium).

Then ^{3}H-thymidine (^{3}H-TdR 0·025 ml, 0·5 μCi/ml, sp.act. 22 Ci/mmol UVVR, Prague) was added to give a final concentration of 2 μCi/ml and the incubation was continued for a further hour. Controls without cytostatics were set up in each series of experiments. All tests were performed in duplicate. ^{3}H-TdR incorporation into DNA was determined by liquid-scintillation counting. The effect of cytostatic drugs is expressed as a reduction in ct/min/10^7 cells in treated cells relative to the controls.

Nuclear morphology in endometrial cancer undergoing progesterone treatment.—Twenty patients with endometrial cancer who were given progesterone as a preliminary therapeutic measure have been investigated.

Tissue samples were obtained at the diagnostic curettage (A sample). All patients were then submitted to progesterone therapy (DEPO Provera, Upjohn S.A., Puurs, Belgium) i.m. on consecutive days to a total amount of 600 mg. On the 8th–10th day after progesterone therapy, several tissue samples (B sample) were obtained, either from curettage before intracavitary radium treatment, or at hysterectomy. All tissue specimens were fixed in methanol/formalin/acetic-acid mixture for 24 h, embedded in paraffin and 4 μm histological section were stained with haematoxylin and eosin. In each specimen (A or B) 150–200 tumour-cell nuclei were studied at random for measurement of the long and short axes, the ratio of the 2 axes being expressed as the "elongation". Secretory conversion of the endometrial cell population is morphologically expressed by shortening of the long axis of the nuclei, thus decreasing the elongation (Epifanova, 1971).

In all these specimens some aspects of cell kinetics were also studied using double labelling with ^{3}H-TdR-^{14}C-TdR, the results of which are published separately (Sirackỳ *et al.*, 1978).

RESULTS AND DISCUSSION

Ploidy

All the cases in our series were characterized before treatment by a wide range of ploidy, with modal chromosome numbers in the diploid or hyperdiploid range, which is characteristic of ascites tumour cell populations in ovarian cancer (Atkin, 1976; Koller, 1972).

"Number of mitoses studied" in Table I refers to samples studied before, during and after cytostatic treatment in sequence. Some of the cases are represented by only 2 samples (before and after treatment) due to inhibition of the ascites proliferation. "Modal chromosome number" refers to the pretreatment sample, and apart from case No. 11 (Fig. 2) no changes in modal chromosome number during cytostatic treatment were detected. In several of these cases, however, distinct changes in ploidy distribution

TABLE I.—*Survey of 12 advanced ovarian cancers with sequential chromosome studies of ascites tumour cells before, during and after cytostatic treatment*

						Ploidy distribution of mitoses											
						Before treatment						After treatment					
No.	Histology	Cytostatic treatment	No. of samples	No. of mitoses studied	Modal number	Near diplo	%	Near tetra	%	Higher ploidy	%	Near diplo	%	Near tetra	%	Higher ploidy	%
1	Adenoca	Leukeran +2×5 mg T.S. 160 i.p.	3	107, 106, 70	59	79	73·8	5	4·6	23	21·6	43	61·4	7	10·1	20	28·5
2	Adenoca	Mannogranol +1×5 mg TS-160 i.p.	2	20, 70	50	19	95·0	1	5·0	—		63	90·0	7	10·0	—	
3	Papilloca	Cytembena	5	98, 50, 36, 47, 112	51	82	83·7	10	10·2	6	6·1	85	75·9	27	24·1	—	
4	Adenoca	Mannogranol +1×5 mg TS-160 i.p.	2	70, 75	41	61	87·1	3	4·4	6	8·5	61	81·3	6	8·0	8	10·7
5	Papilloca	Mannogranol +1×5 mg TS-160 i.p.	2	50, 63	81	9	18·0	29	58·0	12	24·0	14	22·2	45	71·4	4	6·4
6	Papilloca	Cytembena	2	51,78	49	43	84·3	8	15·7	—		61	78·2	17	21·8	—	
*7	Papilloca	Mannogranol +3×5 mg TS-160 i.p.	4	98, 100, 101, 103	43	33	33·7	30	30·6	35	35·7	96	93·2	5	4·8	2	1·9
8	Adenoca	Cytembena	2	63, 68	56	55	87·3	8	12'7	—		62	91·2	6	8·8	—	
9	Adenoca	Mannogranol +1×5 mg TS-160 i.p.	2	72, 54	54	69	95·8	3	4·2	—	50	50	92·6	4	7·4	—	
10	Adenoca	Mannogranol +1×5 mg TS-160 i.p.	2	72, 76	52	55	76·4	9	12·5	8	11·1	60	79·0	11	14·5	5	6·5
†11	Papilloca	Cytembena	5	98, 68, 71, 81, 130	51 46	88	89·8	10	10·2	—		126	96·9	4	3·1	—	
12	Adenoca	TS-160	3	98, 101, 86	62	73	74·5	14	14·3	11	11·2	77	89·5	7	8·1	2	2·4

Total doses (all parenteral applications) during the course of the investigation (3–5 weeks): Leukeran—200 mg, Cytembena—6000 mg, Mannogranol—20·0 g, TS-160—25 mg. (+specified i.p. TS-160 doses).

* See Fig. 1. † See Fig. 2.

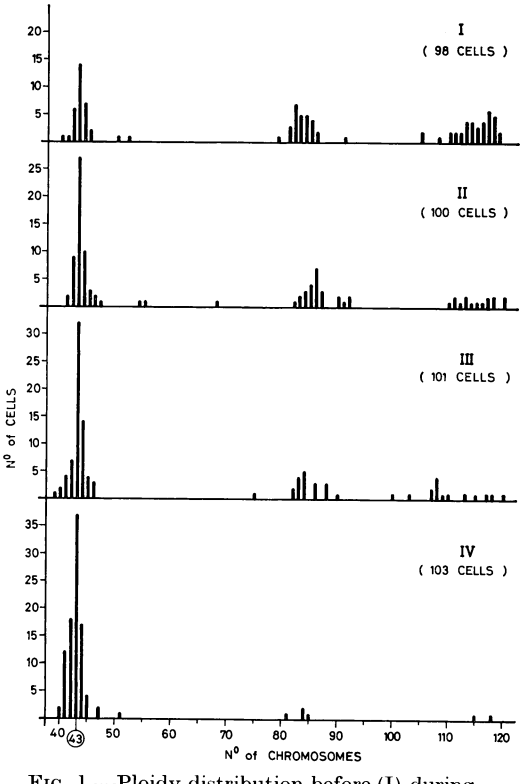

FIG. 1.—Ploidy distribution before (I) during (II, III) and after (IV) cytostatic treatment of tumour No. 7 in Table I.

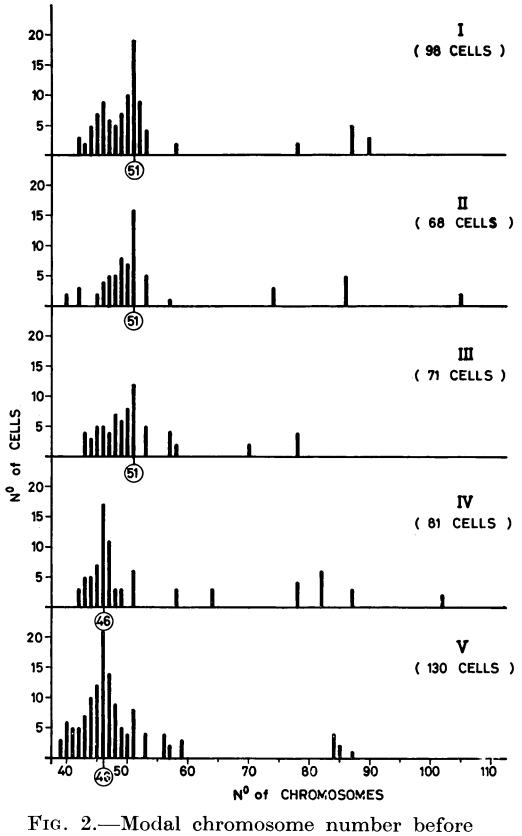

FIG. 2.—Modal chromosome number before (I), during (II, III, IV) and after (V) cytostatic treatment of tumour No. 11 in Table I.

(cases 1, 3, 5, 7 and 12) during cytostatic treatment were evident. This type of chromosomal change seems to demonstrate selective overgrowth of cell clones, those resistant to the cytostatic treatment (Fig. 1 clearly demonstrates this type of change).

One spontaneous change with the age of the ascites is polyploidization (Koller, 1972) which is also to be expected as the result of damage to the mitotic process of cells by alkylating agents (Bishun, 1971; Nasjleti & Spencer, 1967). The ploidy distribution changes in our series are, however, characterized by decrease and/or disappearance of polyploid cells, which suggests increased sensitivity of polyploid clones to cytostatic drugs.

This form of sequential cytogenetic study during the follow-up of a patient (Eicke *et al.*, 1965; Mohr *et al.*, 1966; Siracký, 1969; Visfeldt & Lundwall, 1970) illustrates biological and cytogenetically expressed heterogeneity of the tumour-cell population in sensitivity to cytostatic agents, and contributes to a better understanding of the processes of cell selection in human tumours during treatment. There are, however, great difficulties in the interpretation of such analyses, since most of the cases with effusions are in an advanced stage, with metastatic dissemination and very poor prognosis.

^{3}H-TdR incorporation

In most of the investigated cases, different parts of the same tumour, different metastatic nodes of the same patient, as well as the primary tumour and its ascitic form, differ significantly in their

TABLE II.—*Differences in drug sensitivity of samples from different areas within the same tumour (expressed as ct/min/10^7 cells after uptake of [^3H]-TdR)*

Ca colon

Sample	Control ct/min	Control %	FU ct/min	FU %	MTX ct/min	MTX %	TS ct/min	TS %	VINCR ct/min	VINCR %
1	309	100	289	93·5	90	29·1	77	24·9	212	68·6
2	384	100	256	66·6	80	20·8	44	11·4	52	13·5
3	421	100	312	74·1	487	115·7	95	22·6	7	1·6

Ca sigmae

Sample	Control ct/min	Control %	FU ct/min	FU %	MTX ct/min	MTX %	TS ct/min	TS %	VINBL ct/min	VINBL %
1	296	100	312	105·4	270	91·2	250	84·5	222	75·0
2	322	100	230	71·4	292	90·7	174	54·0	76	23·6

Ca ovary

Sample	Control ct/min	Control %	FU ct/min	FU %	MTX ct/min	MTX %	TS ct/min	TS %
1	457	100	403	88·2	312	68·2	125	27·3
2	397	100	305	76·8	370	93·2	231	58·2
3	524	100	400	76·3	305	58·2	96	18·3

Dose schedule in Tables II, III and IV:

ALK— 0·2 mg/ml
FF— 2·0 mg/ml
FU— 0·5 mg/ml
MTX— 0·05 mg/ml
OXAUR—0.01 mg/ml
TS— 0·1 mg/ml
VINBL— 0·1 mg/ml
VINCR— 0·1 mg/ml

TABLE III.—*Differences in drug sensitivity of samples from primary tumours and their metastases and/or between different metastatic nodes (Expressed as ct/min/10^7 cells after uptake of [^3H]-TdR)*

Advanced Ca ovary with metastatic dissemination

Sample	Control ct/min	Control %	TS ct/min	TS %	MTX ct/min	MTX %	FU ct/min	FU %
Primary tumour 1	704	100	368	52·2	693	98·4	480	68·2
Primary tumour 2	652	100	310	47·5	699	107·2	334	51·2
Metastatic node	786	100	249	31·7	522	66·4	449	57·1

Advanced Ca ovary—metastases omenti maioris

Sample	Control ct/min	Control %	TS ct/min	TS %	MTX ct/min	MTX %	FU ct/min	FU %
Met. 1	801	100	165	20·6	1110	138·6	553	69·0
Met. 2	661	100	157	23·7	367	55·5	221	33·4
Met. 3	604	100	251	41·6	970	160·6	377	62·4

sensitivity to the same cytostatic treatment *in vitro* (Tables II, III, IV). These variations in tissue specimens might be explained by local nutritional conditions, by variations in the proliferation rate and/or by variations in the amount of necrosis in different parts of the tumour. These factors ought to be responsible for differences in the incorporation of the ^{3}H-TdR in control specimens, but no large differences were found between controls. It can be assumed, therefore, that the differences in sensitivity to cytostatic treatment are due to the presence of 2 or more clones of tumour cells differing in their biological characteristics.

TABLE IV.—*Differences in drug sensitivity of primary tumours and their ascitic tumour-cell population (expressed as ct/min/10^7 cells after uptake of [^3H]-TdR)*

Advanced gastric cancer with ascites

	Control		ALK		FU		MTX		FF	
Sample	ct/min	%	ct/min	%	ct/min	%	ct/min	%	ct/min	%
Tumour	399	100	432	108·2	213	53·4	195	48·9	384	96·2
Ascites	474	100	198	41·7	144	30·3	96	20·3	108	22·8

Advanced Ca ovary with ascites

	Control		FU		MTX		TS		OXAUR	
Sample	ct/min	%	ct/min	%	ct/min	%	ct/min	%	ct/min	%
Tumour	378	100	371	92·2	668	176·7	211	55·8	309	81·8
Ascites	459	100	670	146·0	295	64·2	236	51·4	228	49·7

N clear morphology

Clinical experience has shown that endometrial cancers are hormone (progesterone) responsive in a considerable percentage of cases (Kelley & Baker, 1965; Smith *et al.*, 1966). Several studies have also demonstrated secretory conversion, mostly in highly differentiated cancers, manifesting itself by characteristic changes in nuclear morphology as a result of progesterone treatment (Hustin, 1976; Martinez-Manaton *et al.*, 1975). Secretory conversion implies loss of proliferative

TABLE V.—*% of cells from 20 endometrial cancers with mean length of nucleus <12 μm, before and after progesterone treatment*

No.	Degree of differentiation	Before treatment	After treatment
1	H	58·3	85·0
2†	H	45·7	84·2
3	H	54·6	73·6
4	H	68·1	84·0
5	H	58·7	83·1
6	H	53·5	81·7
7	H	60·0	84·1
8	M	56·4	66·8
9	M	58·6	63·4
10	M	55·5	62·9
11	M	57·3	78·0
12	M	53·9	62·4
13	M	58·9	87·8
14*	M	46·9	54·1
15	M	44·3	55·1
16*	M	48·6	56·9
17	P	38·4	54·8
18*	P	43·2	52·0
19*	P	48·8	53·1
20*	P	44·9	51·4

* Separation into 2 distinct cell populations as seen in Fig. 4.

† See Fig. 3.

H=high. M=Moderate. P=Poor.

12 μm was selected as the mean +s.d. for pre-treatment samples.

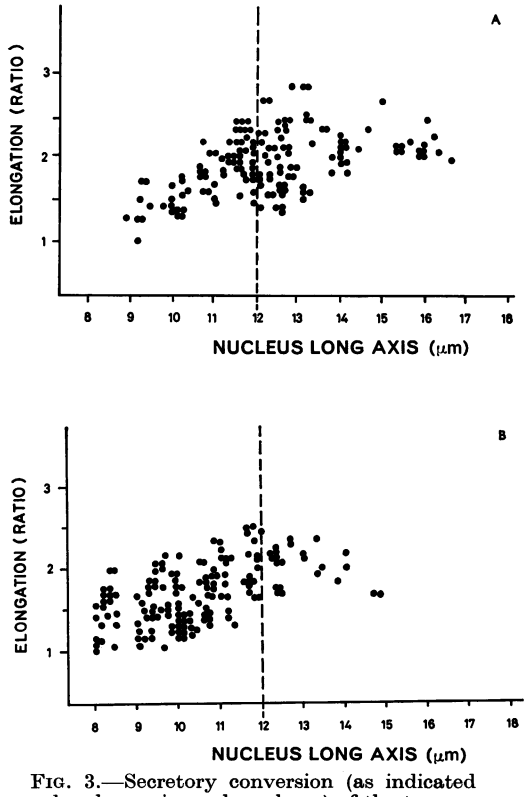

FIG. 3.—Secretory conversion (as indicated by change in nuclear shape) of the tumour cells in a highly differentiated cancer (No. 2 in Table V). A=before and B=after progesterone treatment.

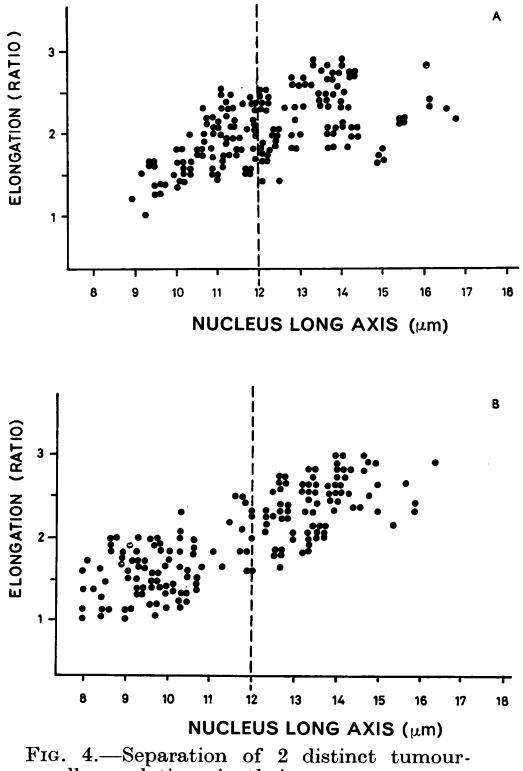

FIG. 4.—Separation of 2 distinct tumour-cell populations in their response to progesterone in a case of poorly differentiated cancer (No. 19 in Table V). A = before and B = after treatment.

capacity and might be a prelethal stage (Epifanova, 1971).

Most of the highly differentiated cancers in our series exhibited significant responses to progesterone, expressed as rising values of potential doubling time in post-treatment specimens (Siracký *et al.*, 1978) as well as showing secretory conversion of a significant fraction of the tumour-cell population (Table V, Fig. 3). In highly differentiated cancers of our series the ratio of long-short axis of nuclei changed from $1{\cdot}74 \pm 0{\cdot}52$ before to $1{\cdot}35 \pm 0{\cdot}41$ after progesterone treatment. The mean length of the long axis was $10{\cdot}38 \pm 2{\cdot}34$ μm before and $8{\cdot}69 \pm 2{\cdot}03$ μm after treatment.

Examples of the changes in nuclear morphology after progesterone treatment are shown in Figs 3 and 4. Table V presents the changes in percentage of nuclei <12 μm long in 20 different endometrial tumours.

In the group of moderately and poorly differentiated cancers, in spite of nearly unchanged values both in proliferation kinetics and nuclear morphology after progesterone treatment, a distinct fraction of the tumour-cell population undergoing secretory conversion could be detected by plotting the nuclear morphology parameters in nomograms (Fig. 4) which clearly demonstrated differences in response to progesterone treatment within the cell population of a single tumour. It appears, however, that recognition of 2 different types of cell population, as seen in Fig. 4B, would be possible only in tumours with about equal amounts of both cell types.

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