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Prolonged G2 Phase of Breast Cancer Cells and Chromosome Damage

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5-bromodeoxyuridine incorporation was studied in metaphase chromosomes from 24 breast tumour specimens, including 23 adenocarcinomas. In these 23 cases, a slow cell cycle was observed, with a long (8 h) G2 phase. This slowing of the cell cycle, which was poorly related to the degree of polyploidy, was significantly related to the number of chromosome anomalies: the cell cycle was particularly slow when many rearranged chromosome were observed. These *in vitro* findings during the first cell cycle cannot easily be transposed to the *in vivo* situation. By analogy with Fanconi anaemia, in which both chromosome lesions and a long G2 phase are detected, a DNA repair defect and/or high DNA mutagenesis might exist in breast cancer cells.
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INTRODUCTION

OF THE solid tumours, breast cancer is one of the most difficult to study cytogenetically beause of the poor growth of breast cancer cells *in vitro* and the frequent occurrence of cases with highly abnormal metaphases, which makes karyotyping almost impossible. Nevertheless, it is possible to identify a number of recurrent anomalies [1, 2]. Several chromosome losses, which are also seen as losses of heterozygosity, suggest that some events of tumour progression are determined by recessive genes, the deletion of one allele unmasking the remaining mutated allele.

To classify the tumours, we have taken advantage of the wide range of chromosome anomalies to differentiate cases with a few from those with many rearranged chromosomes, and also hypodiploid and near diploid from hyperploid cases. We studied chromosome banding by incorporation of 5-bromodeoxyuridine (BrdU) to investigate cell cycle progression [3–9], a method rarely used in cancer cytogenetics. To our knowledge, an anomaly of the cell cycle has been described in a single constitutional disease: Fanconi anaemia [10]. Data from transformed or cancerous cells are not more numerous, and their study is complicated by the possible anomalies in BrdU incorporation which may be related to unusual metabolism of BrdU [11]. One of the particularities of transformed or cancerous cells is their capacity to undergo many successive cycles, without pausing in G0 phase. This may give the false impression that they are cycling quickly. We would like to report that cells from mammary adenocarcinoma arrived in first metaphase *in vitro* have a slow cycle, especially because of a very long G2 phase.

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MATERIALS AND METHODS

The clinicopathological data of the 24 tumours studied are shown in Table 1. The chromosomal data of 6 cases has been reported [12]. All cultures, initiated after biopsy or puncture, were short-term (1–4 days), and BrdU (10 μg/ml) was added to the culture medium from 7 to 72 h before harvesting metaphases. Other cultures, without addition of BrdU, were always done to assess R-banded karyotypes.

The degree of BrdU incorporation was evaluated with a modified fluorescence plus Giemsa technique [9]. When possible, 50 metaphases were analysed for each experimental condition. According to the classification proposed by Couturier and Antoine [13], seven phases or subphases were distinguished: (i) G2, no modification of chromosome staining; (ii) late S (lS),

Table 1. Clinicopathological and cytogenetic details from 23 malignant and 1 benign breast tumour cases

Case/age	TNM	Classification SBR*	WHO	Chromosomes(–)†	Range	Mode	Rearranged chromosomes(–)‡	Analysed metaphases(–)
(A) Near diploid, < 11 rearranged chromosomes								
1/65	T2N0	I	IDC	45.5 (1.3)	41–46	46	4	20
2/68	T2N0	II	IDC	44.0 (1.6)	39–45	45	1	15
3/72	T3N0	I	IDC	43.6 (0.8)	41–44	44	2	14
9/74	T4N0	II	IDC	44.7 (0.6)	43–45	45	2	15
10/63	T2N0	II	IDC/ILC	47.8 (0.8)	45–48	48	2	13
M2/43	T1N0	I	IDC	46.3 (0.5)	46–47	46	4	12
(B) Hypodiploid, > 10 rearranged chromosomes								
26/44	T2N0	II	IDC	41.0 (2.5)	38–47	40	16	10
				74.4 (2.8)	71–79	71		7
35/47	T2N1	II	IDC	38.5 (3.6)	30–43	40	20	10
				71.6 (1.3)	68–74	73		15
54/37	T2N1	III	IDC	38.8 (1.6)	35–40	40	19	22
				57.8 (10.1)	50–75	–		4
55	–	–	–	41.0 (2.3)	37–43	43	23	6
(C) Polyploid, < 11 rearranged chromosomes								
103/44	T2N0	II	IDC	82.7 (4.8)	75–90	–	8	6
107/51	T2N0	III	IDC	76.2 (2.4)	71–79	76	8	10
(D) Polyploid, > 10 rearranged chromosomes								
105/67	T1N0	II	IDC	72.0 (1.9)	69–73	–	22	4
116/56	T2N0	–	MC	61.2 (5.8)	51–58	–	20	6
117/60	T2N0	III	IDC	72.3 (3.5)	68–79	68	20	10
121/34	T2N0	–	MIDC	89.3 (1.5)	87–91	–	12	4
122/71	T3N0	II	IDC	65.6 (1.8)	60–68	66	12	7
123/77	T2N0	II	IDC	57.3 (7.4)	49–64	63	32	8
124/59	–	–	–	61.0 (1.7)	64–70	67	45	8
125/53	–	–	–	71.3 (3.2)	67–75	73	20	7
134/63	T2N0	–	SCC	55.3 (4.8)	51–66	54	24	7
				95.0 (8)	82–104	–		4
136/66	T2N0	III	IDC	59.7 (3.3)	55–64	62	14	15
148/62	T2N0	I	ILC	61.8 (1.6)	59–66	62	27	14
Benign phyllodes tumour								
150/56	–	–	–	46	46	46	0	10

IDC = infiltrating ductal carcinoma, ILC = infiltrating lobular carcinoma, MIDC = micro-invasive ductal carcinoma, MC = mucinous carcinoma and SCC = spindle cell carcinoma.

Cases 124 and 125 were nodal, case 55 pleural metastases and M2 a male patient.

* Bloom and Richardson grading. † Mean (variance). ‡ Best estimate after exclusion of duplicated derivatives.

pale staining of constitutive heterochromatin and of the late replicating X (Fig. 1a); (iii) mid–late S (m–lS), pale staining of latest replicating G-bands in addition to appearance in 1S (Fig. 1b); (iv) mid–S (mS), pale staining of all G-bands in addition to appearance in 1S (Fig. 1c), giving typical R-banding; (v) mid–early S (m–eS), pale staining of latest replicating R-bands in addition to appearance in (iv) (Fig. 1d); (vi) early–S (eS), pale staining of all bands except the earliest replicating R-bands (Fig. 1e); and (vii) G1, pale staining of all chromosomes with, occasionally, a discrete strengthening of constitutive heterochromatin (Fig. 1f). For each analysed metaphase, the chromosome staining indicated the stage at the beginning of BrdU treatment.

RESULTS

Karyotypes

The mean, mode and range of the chromosome numbers, as well as the mean number of different rearranged chromosomes per karyotype are shown in Table 1. The 23 malignant tumours could be classified into four cytogenetic categories. Patient 150, who had a benign phyllodes tumour, had a normal karyotype.

Cell cycles

Results of BrdU incorporation times ranging from 8 to 21 h could be obtained. For 20 cases, a time of 17–18 h was selected (Table 2).

In comparison with normal lymphocytes and fibroblasts (our

Table 2. Distribution of cells in different phases 17–18 h before harvesting metaphases

Chromosome type	A							B			C		
	1*	2	3	9	10	M2	Total(%)	26	54	Total(%)	103	107	Total(%)
Phase													
G2	3	11			2	2	18 (10)	2	15	17 (16)		2	2 (3)
lS	1	5	1		6	1	14 (8)	4	14	18 (17)		0	
m-lS	1	7	5	1	3	3	20 (11)	7	28	35 (33)		3	3 (5)
mS	1	2	22			3	28 (16)	6	20	26 (24)	4	17	21 (35)
m-eS	10	1	13	1		2	27 (15)	3	1	4 (4)	2	15	17 (28)
eS	10		4	2		1	17 (9)		1	1 (1)	2	8	10 (16)
G1	24	3	5	10		14	56 (31)	6		6 (5)	3	5	8 (13)
Total	50	29	50	14	11	26	180	28	79	107	11	50	61

Chromosome type	D													
	26	54	105	116	117	121	122	123	124	125	134	136	148	Total(%)
Phase														
G2	1	4	2		13	–	18		5	16		6	9	74 (18)
lS	2	4			10	1	15	3	2	4	1	7	4	53 (13)
m-lS	9	5	2	4	14	9	15	5	4	2	5	2	9	85 (19)
mS	11	3	1	5	5	6	2	3	2	4	8	2	20	72 (20)
m-eS	7			14	5	1		1	2	2	2		5	39 (13)
eS				15	8	1					1		1	26 (8)
G1	2			7	17	2				6			2	36 (9)
Total	32	16	5	45	72	20	50	12	15	34	17	17	50	385

Cases 26 and 54 are in both categories B and D, because they have one hypodiploid and one hyperploid clone, counted separately.

*Patient no.

data and ref. 13), the cell cycles were generally slow: 66% (486/733) cells needed a time of 17 h or more to pass from mS to metaphase. This slowness was not related to the quality of cell growth *in vitro*, since the proportion was 66% (265 out of 401) for cases with at least 50 analysed metaphases and 67% (221 out of 332) for cases for which less than 50 metaphases could be analysed. The slowness may be related to the degree of ploidy: the proportion was 61% (176 out of 287) for near diploid and hypodiploid (categories A and B) and 70% (310 out of 446) for hyperploid tumours. The excess of cells with a slow cell cycle was significant among hyperploid tumours (χ^2 test, $P < 0.02$). This was exemplified by 2 tumours (cases 26 and 54) in which the hypodiploid clone was slightly faster than the hyperploid clone (Fig. 2).

The cell cycle time was also related to the complexity of chromosome rearrangements. In categories A and C, which have 10 rearranged chromosomes at most, the above proportion was 44% (106 out of 241), whereas it was 77% (380 out of 492) in categories B and D, which have more than 10 rearranged chromosomes per karyotype. This difference was significant ($P < 0.0001$). These results are illustrated in Fig. 3. Comparison of the distributions also indicated a significant difference ($P < 0.0001$).

The slowness of the cell cycle was related to the malignant character of the cells. In case 136, 5 cells had a normal karyotype: their cell cycle was much faster than that of abnormal cells (Fig. 4). In case 55, for which the BrdU incorporation time was 9 h only, the 17 cells with a normal karyotype had a faster cell cycle than the 17 cells with an abnormal karyotype (Fig. 4). The one benign phyllodes tumour (case 150) had a much faster cell cycle than any of the malignant tumours: after BrdU treatment for 17–18 h, all metaphases passed through G1 to metaphase, whereas after a 7 h treatment, 40% of cells went through m–1S or mS to metaphase.

The long duration of G2 contributed greatly to the slowing down of the cell cycle. In the few cases for which BrdU treatment was short (8–9 h) most cells went through G2-M only: case 1 (8 h treatment), 50% (25/50), whereas 50% went through lS and G2-M (Fig. 5); case 35 (8 h), 100% (55 cells); case 9 (9 h), 67% (16/24), whereas 33% went through lS and G2-M; and case 55 (9 h), 76% (13/17), whereas 24% went through lS and G2-M.

The S phase may be only slightly slowed down. Unfortunately, only a single tumour (case 1) could be studied in detail. Since it had a poorly rearranged karyotype, grew very well, and had a relatively fast cell cycle, it may not be representative. The distribution of the various phases was studied every 2 h from 8

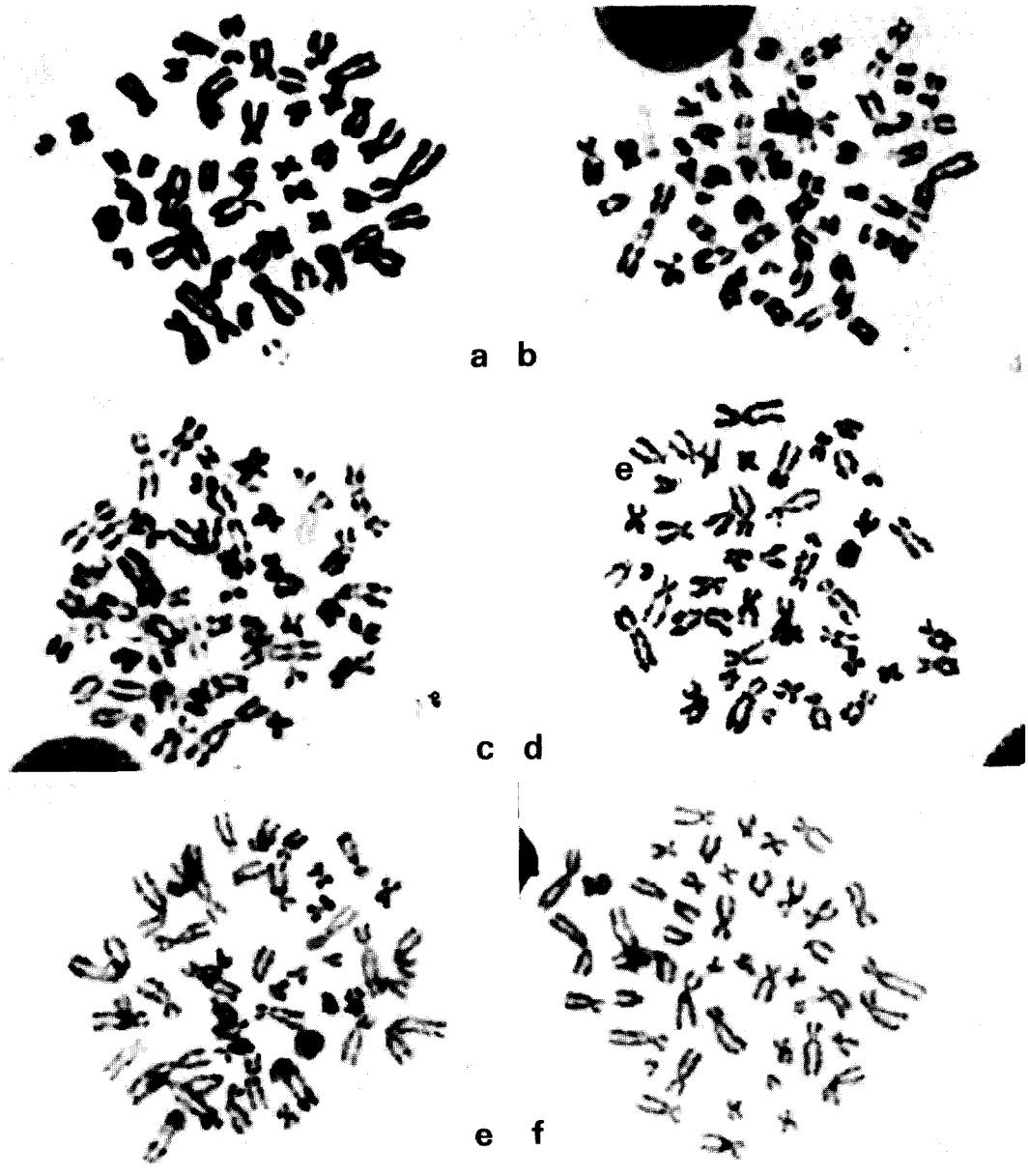

Fig. 1. Metaphases from case 1 with incorporation of BrdU at various times of S phase: (a) = lS, (b) = m–lS, (c) = mS, (d) = m–eS, (e) = eS and (f) = since beginning of S phase or earlier (G1).

to 20 h in 50 metaphases at each time (Fig. 5).The histograms show no block at a given moment of the S phase, since there is no accumulation at a given subphase. Most cells passed through G1 to G2 from 20–18 to 8 h, which indicated an S phase duration of less than 10–12 h.

DISCUSSION

Without exception, our results showed that cells from adenocarcinoma of the breast have a slow cell cycle. In comparison with phytohaemagglutin-stimulated lymphocytes, for which G2-M is about 2–3 h and the S phase about 7–8 h [13], the duration of G2-M was especially slow (about 3-fold longer), whereas the S phase was only 1.5 fold longer. This result from cells during their first cycle *in vitro* cannot be directly transposed to the *in vivo* situation.

Paradoxically, our data cannot be compared to the information provided by the numerous reports with flow cytometry, which focused on DNA ploidy and on the proportion of S phase cells [14–17], but not on G2 phase. Our data (Table 1) showed that all our cases were aneuploid. 16 of 22 tumours passed through endoreduplication. In addition, all underwent chromosome losses and rearrangements. These three events (endoreduplication, losses and rearrangements of chromosomes) are related, and near diploid (our category A) and hyperploid tumours with many chromosomes (our category C) had fewer rearranged chromosomes than hypodiploid and slightly hyperploid (our categories B and D) tumours. Although all these tumours had a slow cell cycle, this slowness was not equally distributed: it was slower in categories B and D than in categories A and C.

The cause of this relation, which we could demonstrate statistically, remains to be elucidated. The cell cycle may progressively slow down when tumours become highly aneuploid and have many chromosome rearrangements. It is also possible that tumours with a slow cell cycle are prone to rearrange

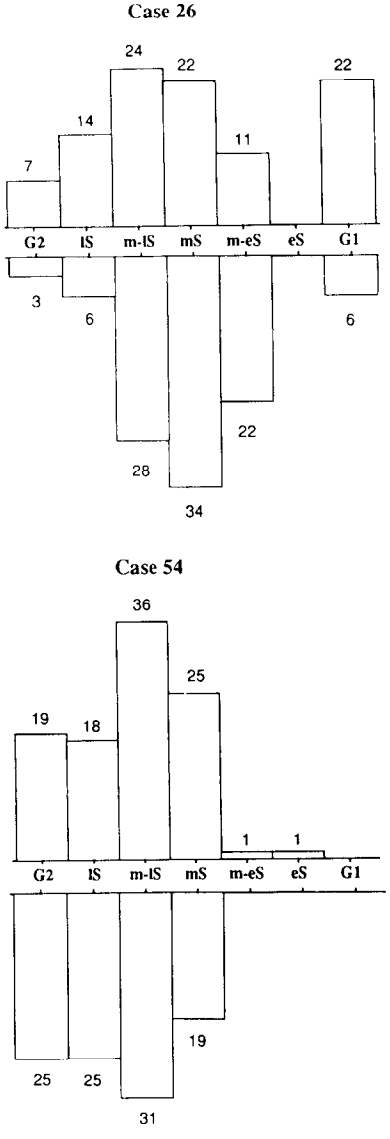

Fig. 2. Percentage distribution of subphases of S, G1 and G2 17–18 h before harvesting metaphases in two cases in which hypodiploid (upper) and polyploid clones (lower) coexisted. Cases 26, 60 metaphases and 54, 95 metaphases.

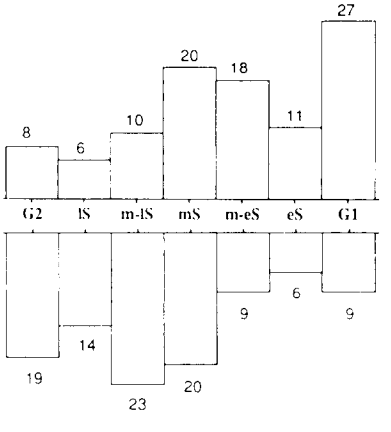

Fig. 3. Percentage distributions of subphases of S, G2 and G1 17–18 h before harvesting metaphases. Categories A and C, 241 cells with few (≤ 10) rearranged chromosomes (upper); categories B and D, 492 cells with many (> 10) (lower).

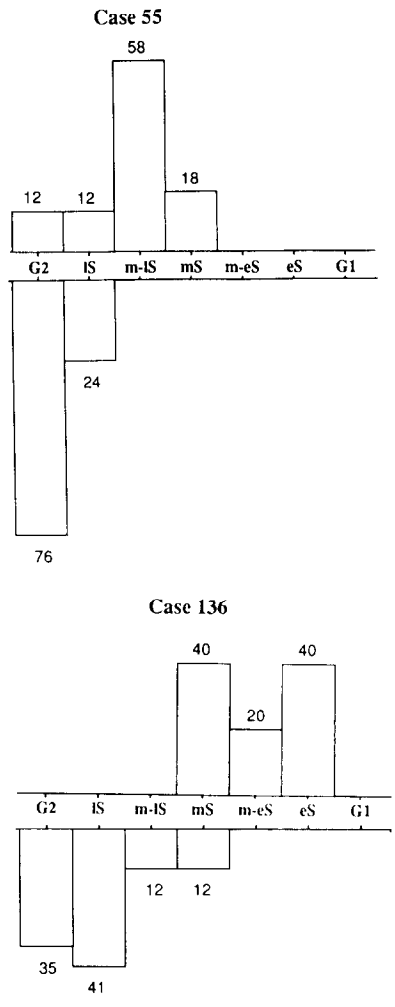

Fig. 4. Percentage distribution of subphases of S and G2 9 h (case 55, top) or 18 h (case 136, bottom) before harvesting metaphases. In both cases, normal (46, XX, upper) coexisted with abnormal cell line (lower).

and lose their chromosomes. Interestingly, however, the duration of the cell cycle may be related to DNA mutations and repair. In Fanconi anaemia, the cell cycle is prolonged, in particular because G2 is about twice as long as in controls [10]. This delay was suppressed by the addition of caffeine, which inhibits postreplication repair [18], during the last hours of culture, but this resulted in an increase in chromosome breakage. It was concluded that Fanconi anaemia cells usually arrive at G2 with many DNA lesions, many of which are repaired during G2, which may be prolonged for this reason. This relation between a high number of rearranged chromosomes and a slow G2 phase in breast cancer cells may also be due to the presence of many DNA lesions occurring during or after replication, of which many are repaired during G2. Unfortunately, we could not repeat these experiments on primary cultures of breast cancer cells because of technical difficulties. In comparison with colorectal tumour cells, which have a faster cell cycle, breast adenocarcinoma metaphases have more chromatid exchanges (radials) [20].

Thus, by analogy with Fanconi anaemia, for which a DNA repair defect is strongly suspected, it can be proposed that breast cancer cells undergo either a defect in DNA repair or a high

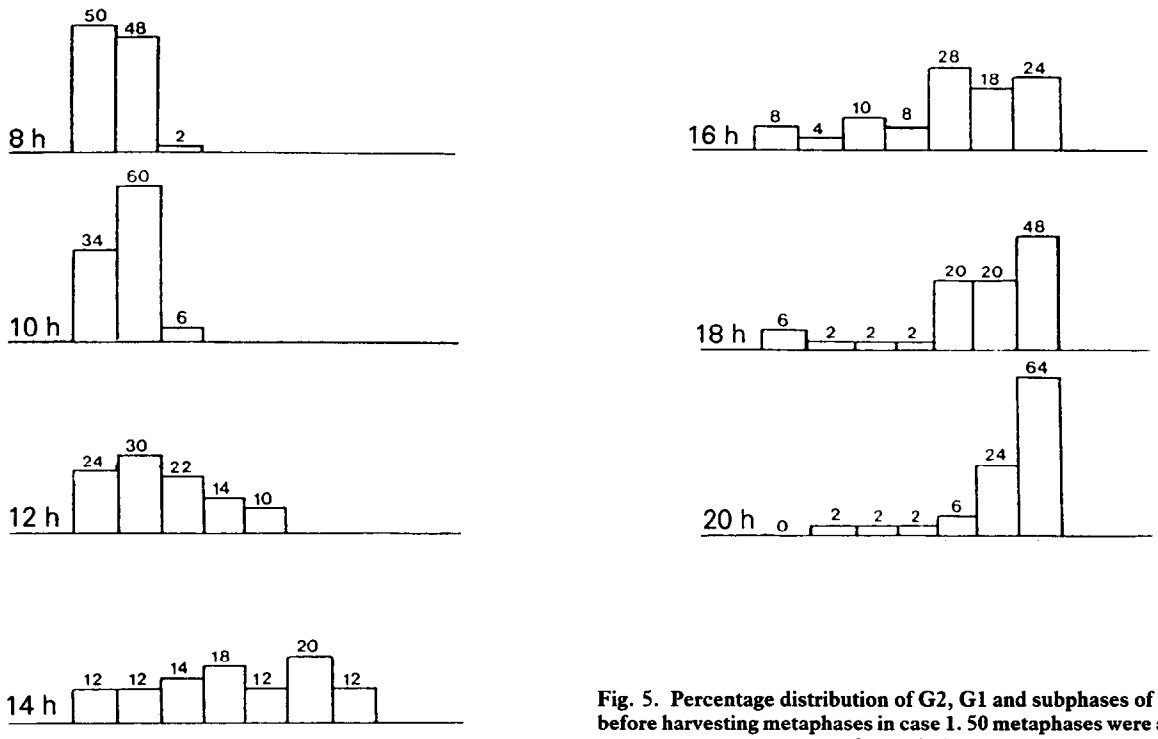

Fig. 5. Percentage distribution of G2, G1 and subphases of S 8–20 h before harvesting metaphases in case 1. 50 metaphases were analysed for each time.

mutagenesis or both, which is possibly responsible for the existence of very abnormal karyotypes and for the slowing of the cell cycle. This anomaly may be principally postreplicative, but any suggestion about similarities in the mechanisms involved in Fanconi anaemia and in breast cancer is speculative.

Our finding of unusual cell cycles of breast cancer cells, if confirmed by *in vivo* studies, may give information about the response to antineoplastic drugs that affect the S phase.

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