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SYNCHRONIZATION CULTURE OF AMNIOTIC FLUID CELLS USING EXCESS THYMIDINE BLOCK FOLLOWED BY DEOXYCYTIDINE RELEASE AND ITS APPLICATION TO HIGH-RESOLUTION BANDING ANALYSIS OF CHROMOSOMES

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Summary We describe a simple synchronization culture technique of amniotic fluid (AF) cells to yield many earlier mitotic divisions with extended chromosomes. AF cell samples obtained by amniocentesis were cultured in the usual manner. Thirty hours after the first subculture, they were exposed to excess thymidine (0.5 mM). This cell cycle block was released by adding deoxycytidine (10 μM) 18 hr after synchronization. At exactly 7.5 hr after the release, the cultures were treated with Colcemid (0.02 μg/ml) for 20 min then harvested. The mitotic index and the ratio of cells in the earlier mitotic stages were much higher in the synchronized cultures than in the control cultures. The same favorable effects were obtained also in AF cell cultures by combining this technique with ethidium bromide or actinomycin D treatment. The technique was less toxic to the cells, and was simple and reproducible. It was successfully applied to prenatal cytogenetic diagnosis of 2 families with a subtle inherited chromosome abnormality, so it is recommended for high-resolution banding analysis of AF cells and possibly chorionic villus samples.

Key Words synchronization culture, excess thymidine, amniotic fluid cell, prenatal diagnosis, high-resolution banding analysis

INTRODUCTION

Recent advances in culture technique provided a valuable tool for improving band resolution and precision of chromosome analysis. Several methods of synchronization culture in peripheral blood lymphocytes by which many cells in early mitotic stages can be collected have been reported, including methotrexate block with subsequent thymidine release (Yunis, 1976) and excess thymidine block followed by changing the growth medium (Viegas-Péquignot and Dutrillaux, 1978) or by adding deoxycytidine (Wheater and Roberts, 1987). Adding certain chemical

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agents interfering with normal chromosome contraction, such as actinomycin D (AMD) (Yunis, 1981), ethidium bromide (EB) (Ikeuchi and Sasaki, 1979), acridine orange (Mastubara and Nakagome, 1983) and 5'-bromo-2-deoxyuridine (BrdU) (Shah *et al.*, 1983), to cultures at the G_2 stage also resulted in a dramatic increase in early mitotic cells.

The use of these methods in cytogenetic analysis has led to the detection of many cases with *de novo* or inherited subtle chromosome abnormalities that might not have been noticed by the usual banding techniques alone (Schwartz and Palmer, 1984; Latos-Bielenska and Hameister, 1988). Thus, there is an increasing demand for high-resolution banding analysis in prenatal diagnosis. Although attempts have been made to obtain early mitotic figures in amniotic fluid (AF) cells and fibroblasts using AMD (Yu *et al.*, 1981), EB (Jäger and Kuhn-Schlage, 1984) or BrdU (Cheung *et al.*, 1985), the results were not always satisfactory because of low mitotic index or poor chromosome spreading.

Synchronization by excess thymidine has not yet been applied to AF cell cultures despite its low toxicity to cells. In this report, we describe a simple synchronization technique for AF cell cultures using excess thymidine block followed by deoxycytidine release, which can lead to a yield of many early mitotic figures of good quality. Furthermore, this technique, combined with AMD or EB treatment, appeared to be very effective in inducing more finely subdivided bands in chromosomes.

MATERIALS AND METHODS

Cell culture. AF cells from karyotypically normal fetuses were used in the present experiments. Those obtained by amniocentesis at 17 to 19 weeks of gestation were seeded into 25 cm^2 plastic flasks containing Chang's medium or Eagle's MEM supplemented with 20% fetal calf serum, L-glutamine (30 mg/dl) and kanamycin (100 μg/ml). The cells were incubated in 5% CO_2 at 37°C. The culture medium was changed twice a week using the supplemented MEM after primary colonies were established. When sufficient cell growth was attained, subcultures were made using 0.25% trypsin (1: 250 trypsin, Difco, diluted in PBS with 0.5 mM EDTA, pH 7.2) with a split ratio of 1: 2 or 3 After the subculture, only fibroblast-like cells survived, and those cells were subjected to the following procedure.

Synchronization procedure. Thirty hours after subculture, thymidine was added to the culture (final concentration, 0.5 mM). The thymidine block was released after 18 hr of synchronization by adding deoxycytidine (final conc. 10 μM). At an appropriate time after the release, the cultures were exposed to Colcemid (0.02 μg/ml) for 20 min then harvested. The culture medium was not changed throughout the synchronization procedure.

Cell harvest, slide preparation, and G-banding. AF cells were collected by trypsinization. Hypotonic treatment was performed with 0.075 M KCl for 30 min at 37°C. After centrifugation, the supernatant was poured off, and the cell pellet

was completely resuspended in a residual hypotonic solution and fixed with Carnoy's solution (3:1 methanol/acetic acid). The first 1.0 ml of fixative was added drop by drop with constant agitation. The fixation was done at least three times before slide preparation. A small volume of the final cell suspension was dropped onto a dry glass slide from a height of 60 cm. The slides were dried in an incubator at a humidity of 50–60% at 37°C. Preparations aged 3–7 days were subjected to G-banding by a modified method of Seabright (1971).

Analysis. To determine an optimal culture time after the deoxycytidine release, parallel cultures established from the same AF cell sample were treated with Colcemid at intervals of 30 min from 5 0 to 9.5 hr after the release. The optimal culture time obtained was used in the following experiments.

The mitotic index and the distribution of mitotic stages were estimated from a count of 5,000 cells and of 60 or more mitotic figures, respectively, for each sample. Based upon the staging proposed by Yunis *et al.* (1977), we subdivided the mitotic process into 5 phases: prophase (corresponding to the stage of about 850 or more bands per haploid set), prometaphase (corresponding to the stage of about 550 bands), early metaphase, mid-metaphase (corresponding to that of less than 400 bands) and others including anaphase, telophase and unanalyzable ones. In addition, the state of chromosome spreading was assessed by counting the number of overlapping chromosomes per diploid cell from a total of 50 mitotic plates in each sample. For each experiment, a parallel culture, harvested 48 hr after subculture with exposure to Colcemid (0.1 μg/ml) for 2 hr, served as a control.

The above synchronization technique was further combined with EB (Jäger and Kuhn-Schlage, 1984) or AMD plus vinblastine (Yu *et al.*, 1981) treatment. The results of these combined techniques were compared with those of the original techniques using EB and AMD plus vinblastine treatment. In the combined methods, these agents were added to the culture 1 hr before harvest.

Finally, this synchronization technique was applied to the prenatal diagnosis in two families with a subtle inherited chromosome abnormality.

Synchronization experiments using the single and combined methods were performed on AF cell cultures at the first and the 3rd to 4th passage. The chi-square test and Wilcoxon rank sum test were used for statistical analysis.

RESULTS

Optimal culture time after the release

The change in mitotic index (MI) of consecutive parallel cultures after the deoxycytidine release in 3 different AF cell samples is shown in Fig. 1. In each sample, the MI increased gradually from 6.0–6.5 hr after the release, reached a peak value at 7.5 hr then decreased gradually, indicating that starting the harvest 7.5 hr after the release was optimal. With culture time beyond 8 hr, the proportion of cells in anaphase and telephase increased markedly.

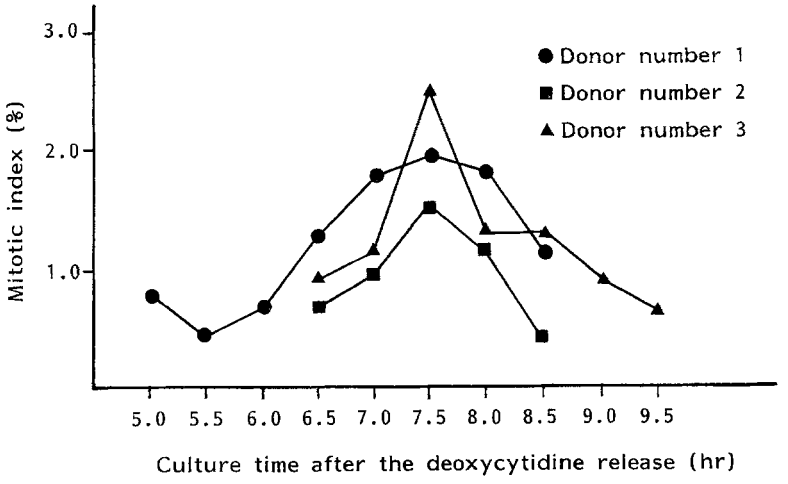

Fig. 1. Change in mitotic index of parallel cultures after the deoxycytidine release.

Table 1. Effects of the synchronization technique on mitotic index and distribution of mitotic stage.

Donor number	Procedure[a]	Mitotic index (%)	Number (%) of mitotic stage						χ^{2} [b]
			Prophase	Prometa-phase	Early metaphase	Metaphase	Others	Total	
1	C	0.93	3(2.1)	8(5.7)	28(19.9)	87(61.7)	15(10.6)	141	57.5
	S	1.83	24(16.6)	35(24.1)	40(27.6)	36(24.8)	10(6.9)	145	
2	C	0.88	0(0)	1(0.8)	14(11.7)	99(82.5)	6(5.0)	120	66.8
	S	1.06	4(3.3)	22(18.2)	44(36.4)	39(32.2)	12(9.9)	121	
3	C	0.65	0(0)	2(2.4)	11(13.4)	56(68.3)	13(15.9)	82	46.8
	S	1.68	2(2.2)	22(24.2)	34(37.3)	21(23.1)	12(13.2)	91	
4	C	0.50	0(0)	1(0.9)	14(12.4)	78(69.0)	20(17.7)	113	40.8
	S	0.89	1(0.8)	17(13.2)	48(37.2)	52(40.3)	11(8.5)	129	
5	C	0.67	0(0)	2(1.8)	12(10.5)	81(71.0)	19(16.7)	114	43.3
	S	1.29	0(0)	17(14.7)	43(37.1)	50(43.0)	6(5.2)	116	

[a] C, parallel control culture; S, culture by excess thymidine. [b] The χ^{2} values indicate highly significant differences (df=4, $p<0.001$) in all the donors examined.

Effects of the synchronization technique on the MI and distribution of the mototic stage (Table 1)

The MI of the synchronized cultures harvested 7.5 hr after the deoxycytidine release ranged from 0.89 to 1.83% in 5 AF cell samples, while those of the control culture were from 0.50 to 0.93%. The MI values of the synchronized culture were 1.8 to 2.6 (mean, 2.2) times higher than those of the control culture. The distribution of the mitotic stage was also clearly altered by the synchronization procedure.

The proportion of cells in earlier mitotic stages such as prophase, prometaphase and early metaphase was 51.2 to 68.3% in the synchronized culture, but it was only 8.1 to 27.7% in the control culture. In all 5 samples, the ratio was significantly higher in the synchronized culture than in the control culture. Representative mitotic figures in each of the synchronized and control cultures are presented in Fig. 2.

Combination of the synchronization technique with EB or AMD treatment (Tables 2 and 3)

The combination of our technique with EB and AMD treatment increased the MI of AF cell cultures 1.4 to 4.4 (mean, 2.5) times and 1.4 to 3.5 (mean, 2.2) times as much as with only the respective procedures. In the samples of donors Nos. 7 and 8, more cells in the earlier mitotic stages were collected by the combined methods than by the single procedures. In another sample (donor No. 6), although there was no significant difference in the distribution of mitotic stage between the combined and single methods, the proportion of cells in prophase and prometaphase was greater in the cultures using the two combined techniques. Earlier mitotic figures in cultures with both the combined methods and single procedures are shown in Figs. 3 and 4.

Effects of the synchronization procedure on chromosome spreading

The distribution of the number of overlapping chromosomes per diploid cell in 3 AF cell samples is given in Table 4. In 2 out of the 3 samples, there were significantly fewer overlapping chromosomes in the synchronized cultures. But in

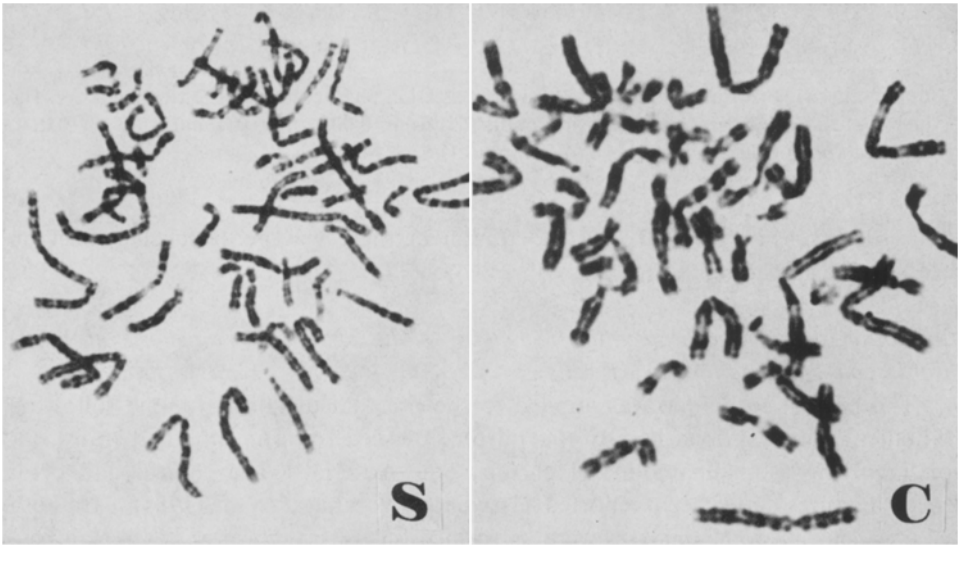

Fig. 2. Representative mitotic figures in the synchronized (S) and control (C) cultures.

Table 2. Effect of the combined technique with ethidium treatment on mitotic index and distribution of mitotic stages.

Donor number	Procedure[a]	Mitotic index (%)	Number (%) of mitotic stage						χ^2 [b]
			Prophase	Prometa-phase	Early metaphase	Metaphase	Others	Total	
6	E	0.41	0(0)	5(4.0)	46(36.5)	55(43.6)	20(15.9)	126	7.7
	S+E	1.79	3(2.3)	14(10.7)	47(35.9)	50(38.2)	17(13.0)	131	
7	E	0.85	2(1.4)	12(8.4)	61(41.8)	60(41.1)	9(7.5)	146	15.0
	S+E	1.38	13(9.0)	23(15.9)	49(33.7)	46(31.7)	14(9.7)	145	
8	E	0.72	1(0.7)	5(3.7)	41(30.4)	68(50.4)	20(14.8)	135	12.3
	S+E	0.98	6(4.8)	13(10.4)	44(35.2)	52(41.6)	10(8.0)	125	

[a] E, ethidium bromide treatment alone; S+E, synchronization plus ethidium bromide treatment. [b] The χ^2 values indicate significant differences in donors 7 (df=4, $0.001<p<0.01$) and 8 (df=4, $0.01<p<0.05$), but not significant in donor 6 (df=4, $p>0.1$)

Table 3. Effect of the combined technique with actinomycin D treatment on mitotic index and distribution of mitotic stage.

Donor number	Procedure[a]	Mitotic index (%)	Number (%) of mitotic stage						χ^2 [b]
			Prophase	Prometa-phase	Early metaphase	Metaphase	Others	Total	
6	A	0.15	0(0)	14(23.0)	27(44.2)	8(13.1)	12(19.7)	61	5.3
	S+A	0.53	5(4.2)	33(27.5)	51(42.5)	18(15.0)	13(10.8)	120	
7	A	0.43	4(2.9)	15(10.9)	48(35.1)	61(44.5)	9(6.6)	137	14.3
	S+A	0.59	12(8.2)	23(15.6)	56(38.1)	38(25.9)	18(12.2)	147	
8	A	0.46	2(1.6)	14(11.3)	42(33.8)	40(33.1)	25(20.2)	124	11.1
	S+A	0.82	8(6.1)	28(21.2)	42(31.8)	40(30.3)	14(10.6)	132	

[a] A, actinomycin D treatment alone; S+A, synchronization plus actinomycin D treatment. [b] The χ^2 values indicate significant differences in donors 7 (df=4, $0.001<p<0.01$) and 8 (df=4, $0.01<p<0.05$), but not significant in donor 6 (df=4, $p>0.1$).

cultures combined with EB or AMD treatment there was no beneficial effect on chromosome spreading.

Application of our synchronization technique to the cytogenetic diagnosis of subtle chromosome abnormalities in AF cells

The present technique was applied to the prenatal diagnosis for the following 2 families. Their infants had 4p− syndrome derived from an inherited translocation involving a small amount of chromosome material. The clinical and cytogenetic features have been reported elsewhere (Narahara *et al.*, 1984). In both cases, the chromosome analyses were performed on AF cell cultures at the 2nd passage.

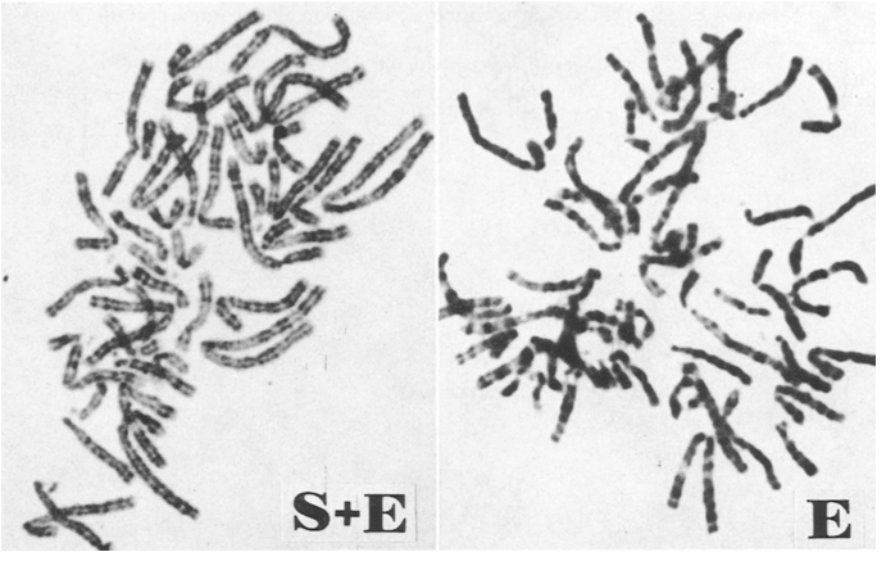

Fig. 3. Earlier mitotic figures in cultures with the synchronization combined with ethidium bromide treatment (S+E) and the single ethidium bromide treatment (E).

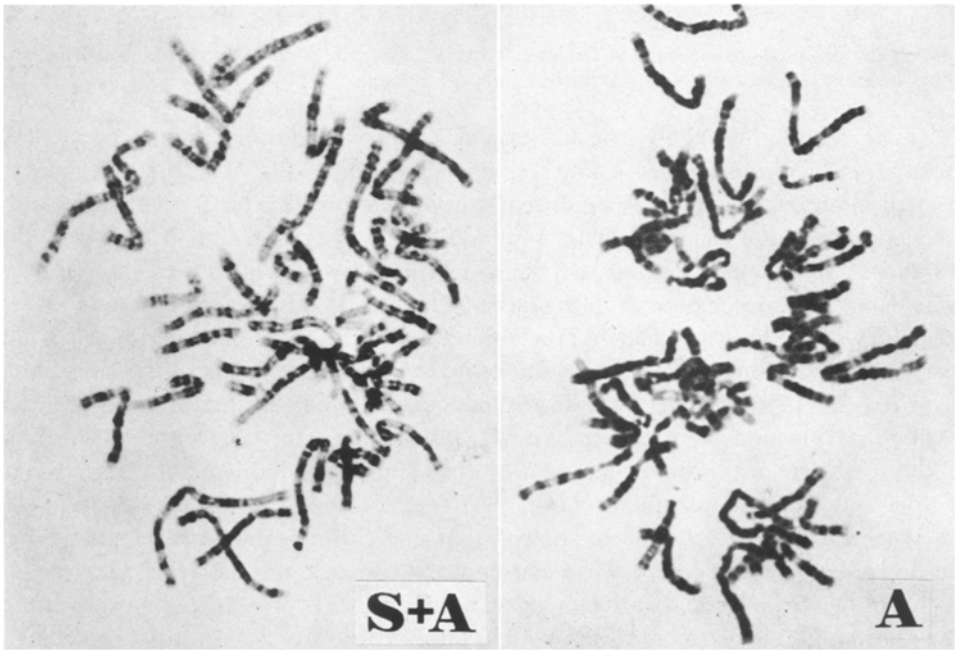

Fig. 4. Earlier mitotic figures in cultures with the synchronization combined with actinomycin D treatment (S+A) and the single actinomycin D treatment (A).

Table 4. Effect of the synchronization technique on chromosome spreading.

Donor number	Procedure[a]	Number of overlapping chromosomes per diploid cell				p value[b]
		0-5	6-11	11-20	21 or more	
6	C	4	10	11	25	NS[c]
	S	12	9	8	21	
	E	7	7	7	29	NS
	S+E	3	5	14	28	
	A	1	4	5	40	<0.01
	S+A	3	11	10	26	
7	C	6	7	17	20	<0.05
	S	10	11	16	13	
	E	1	3	16	30	NS
	S+E	5	11	16	18	
	A	1	9	15	25	NS
	S+A	5	6	14	25	
8	C	1	1	10	38	<0.01
	S	7	5	14	24	
	E	1	0	8	41	NS
	S+E	1	3	10	36	
	A	0	0	2	48	NS
	S+A	0	0	2	48	

[a] Abbreviations used are the same in Tables 1, 2 and 3. [b] Statistical analysis was performed with Wilcoxon rank sum test. [c] No statistically significant difference.

Case 1. In this family, the father had a balanced translocation between the short arms of chromosomes 4 and 18, t(4;18)(p15.2;p11.2). The first pregnancy resulted in an infant with 4p− syndrome, who died soon after birth. In the second pregnancy, prenatal diagnosis with amniocentesis was carried out at 18 weeks of gestation. Cytogenetic analysis of the synchronized AF cell culture showed the same balanced translocation as in the father (Fig. 5). It was decided to continue the pregnancy and a healthy female infant was born at term. Chromosome analysis on her peripheral blood lymphocyte culture showed the above balanced translocation.

Case 2. In this family, the father had an inverted insertional translocation of the distal portion of the short arm of chromosome 4 into the terminal portion of the long arm of chromosome 9, t(4;9)(p15.32p16.3;q34.3). At the 16th week of the 2nd pregnancy, amniocentesis was performed. Cytogenetic analysis of the synchronized AF cell culture showed that one chromosome 4 was replaced by the derivative chromosome 4 while chromosomes 9 were normal (Fig. 6). At 20 weeks of gestation, the pregnancy was terminated by prostaglandin induction. The aborted fetus was found to have craniofacial features typical of 4p− syndrome. The karyotypic abnormality was confirmed on prometaphasic cells obtained from the fetal skin culture.

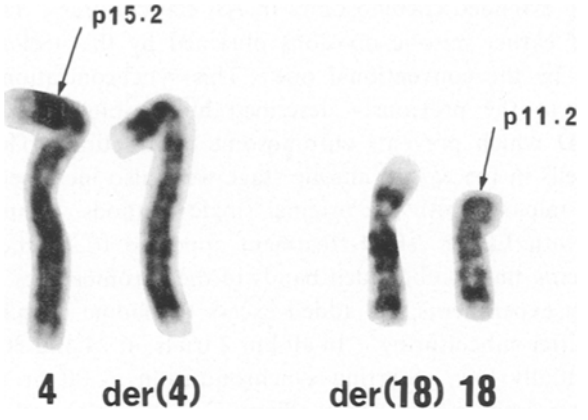

Fig. 5. Partial G-banded karyotype of Case 1 in the synchronized AF cell culture. Arrows indicate breakpoints.

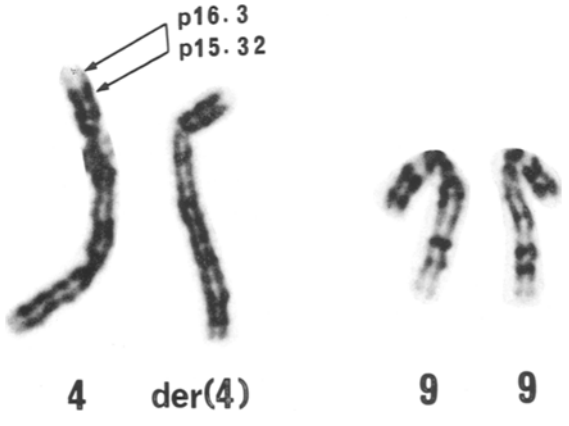

Fig. 6. Partial G-banded karyotype of Case 2 in the synchronized AF cell culture. Arrows indicate breakpoints.

DISCUSSION

Excess thymidine supplemented in a culture is converted to thymidine triphosphate within the cells and inhibits ribonucleotide reductase that catalyzes the reduction of cytidine diphosphate to deoxycytidine diphosphate. The consequent depletion of the deoxycytidine triphosphate pool disturbs the DNA synthesis and accumulates cells in the S-phase (Xeros, 1962). This cell-cycle block can be released either by replacing the thymidine-rich medium with a fresh normal medium or simply by adding affluent deoxycytidine to the culture (Fox *et al.*, 1980).

The present study has demonstrated that the synchronization technique by excess thymidine block followed by deoxycytidine release is practical as well as

useful in providing extended chromosomes in AF cell cultures. The mitotic index and proportion of earlier mitotic divisions obtained by this technique was much higher than those by the conventional one. This synchronization technique was further applicable to the previously described high-resolution banding methods using EB or AMD which prevents chromosome contraction. The mitotic index and the ratio of cells in the earlier mitotic stage were also increased with the combined technique compared with the original single methods. Thus, our synchronization coupled with EB or AMD treatment appeared to be the most effective procedure of inducing finely subdivided bands in the chromosomes of AF cells.

In preliminary experiments, we added excess thymidine to cultures at 12, 24, 30, 36, and 48 hr after subculturing. In all but 2 trials, at 24 and 30 hr, the mitotic index was substantially low. Starting synchronization at 30 hr after subculture in this study was pertinent in view of the efficiency in collecting cells on exponential growth and the time consideration in laboratory work.

The optimal culture time from the deoxycytidine release to harvest for AF cells was 7.5 hr, which differed much from that (5 hr) for lymphocytes (Wheater and Roberts, 1987). The peak of the mitotic index in the consecutive cultures did not occur abruptly but rather gradually. This suggested that the excess thymidine block may not affect a specific short period of cell cycle, *i.e.*, G_1/S border as seen by methotrexate block (Calabresi and Parks, 1985), but may involve a relatively long period in the S-phase. This speculation was supported by the finding that a part of the lymphocytes treated with excess thymidine synchronization and subsequent BrdU incorporation showed G-banding patterns instead of R-banding patterns (Viegas-Péquignot and Dutrillaux, 1978).

No disadvantages were evident in the synchronization procedure. The study of the effects of the synchronization on chromosome spreading demonstrated that the state of spreading was rather improved in most of the samples. Although it has been suggested by Sutherland *et al.* (1985) that excess thymidine induced folic acid-sensitive fragile sites, there was no apparent increase of chromosome or chromatid gaps and breaks in at least 3 samples of synchronized AF cell cultures (data not shown). However, other unfavorable effects of excess thymidine remain to be determined with more AF cell samples.

In AF cell cultures with the synchronization technique, it required at least 2 weeks from the amniotic puncture to the completion of the chromosome analysis. Recently, chorionic villus sampling (CVS) in the first trimester of pregnancy has become a safe and effective technique for early prenatal diagnosis of cytogenetic abnormalities (Rhoads *et al.*, 1989). Although there is a little higher risk of ambiguous diagnosis and fetal loss in CVS than in amniocentesis, pregnant women prefer CVS to amniocentesis because of the less intense feelings of guilt when electing to terminate the pregnancy. In this context, the application of the synchronization technique to chorionic villi may be a subject for future study.

At least two types of cells, fibroblast-like and epithelial cells, are seen in primary colonies of AF cell cultures. The latter cells cannot usually be subcultured. The

present technique has its limitation in cultures where colonies of epithelial cell type predominate. Recently, Deng and Niikawa (1989) have reported that the application of excess thymidine synchronization to short term cultures of chorionic villus samples without passage resulted in a high yield of extended chromosomes. In our laboratory, an attempt to synchronize and harvest AF cell cultures *in situ* is now in progress.

In conclusion, our technique in AF cell cultures is very simple and reproducible. Adding thymidine 30 hr after subculture and deoxycytidine 7.5 hr before harvest is a major task. There is no need to change the culture medium for release of the cell cycle block. As successfully applied to the diagnosis of the subtle chromosome abnormality in the 2 fetuses, this technique is generally recommended for prenatal cytogenetic diagnosis of any case where confirmation of chromosome abnormality has to await high-resolution banding analysis.

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