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Brief Communication

Improved Methods of Direct and Cultured Chromosome Preparations from Chorionic Villous Samples

MING-TSUNG YU,[1] CHIH-YU YU,[2] CHUAN-XING YU,[3] JACK MAIDMAN,[4] AND DOROTHY WARBURTON[3,5]

SUMMARY

A new method is described for preparing direct mitotic chromosome spreads from chorionic villous samples, which has resulted in sufficiently high yields of well-banded metaphases to permit a complete standard chromosomal diagnosis in 20 of 20 cases. A method of establishing monolayer cultures from this material that can be harvested from 3 to 7 days after initiation is also presented.

INTRODUCTION

There would appear to be several advantages to using "direct" preparations rather than monolayer cultures for chromosome analysis from chorionic villous samples: increased speed of diagnosis, decreased cost of medium and other supplies, and probably a smaller chance of observing maternal rather than fetal chromosomes. However, the inconvenience of the work scheduling, the low yield of mitotic figures, and the often unsatisfactory quality of the chromosome preparations have led many laboratories to adopt culture techniques as their preferred or even sole method of analysis.

We describe here a method for making direct preparations that produces sufficient numbers of excellent quality banded metaphase figures to make cul-

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[1] Research Foundation for Mental Hygiene, New York State Psychiatric Institute, New York, N.Y.
[2] Presbyterian Hospital in the City of New York, New York, N.Y.
[3] Department of Human Genetics and Development, Columbia University, New York, N.Y.
[4] Department of Obstetrics and Gynecology, Columbia University.
[5] Department of Pediatrics, Columbia University.

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tured analysis unnecessary in almost every case. We also have developed a culture method that may allow harvesting of cells for chromosome analysis after only 3–4 days.

MATERIALS AND METHODS

Chorionic villous sampling has been attempted in 60 volunteers undergoing elective termination of pregnancy at 7–10 weeks, using a Portex catheter for the first 40 cases and a modification of this catheter for the last 20 cases. Successful villous biopsies of 10 mgs or more were obtained in 40 out of the total 60 attempts and in 20 out of 20 of the last cases. Villi are taken into F-10 medium containing 1 USP unit of sodium heparin per ml. In most cases both direct and cultured methods were attempted. In the early cases we had limited success using the published direct methods. Mitotic figures tended to be sparse and often incomplete, and chromosomes were short and poorly banded. However, in the last 20 biopsies we have been able to perform complete standard chromosome analyses of 20 well-banded cells, using the direct method described below. The method was developed using villi from complete specimens of elective pregnancy terminations of 7–10 weeks. We have also modified our culture procedure, with a decrease in the average time required for culture from 10 days to 5 days.

Direct Method

(1) Carefully dissect villi free of all decidual material under a dissecting microscope in F-10 medium with 20% fetal calf serum and antiobiotics. If there is a choice, choose villi showing the most budding for direct preparations. Dissection may take a considerable length of time; if pH changes to alkaline, incubate in 5% CO_2 until F-10 regains it's normal pH.

(2) Add villi to sterile tube containing 2 cc F-10 medium with 20% fetal calf serum and 2 cc Chang's medium. Incubate at 37°C in 5% CO_2 atmosphere for 5–8 hrs (until end of working day). Keep cap of tube loose during incubation.

(3) Tighten cap and refrigerate specimen in tube overnight at 5°C.

(4) In the morning, loosen cap and incubate at 37°C, 5% CO_2, for approximately 5½ hrs. Add Colcemid, four times the usual concentration (0.4 μg/ml), for ½ hr before harvest.

(5) Centrifuge briefly, remove supernatant, and add 1% sodium citrate. Leave at room temperature for 1 hr.

(6) Centrifuge, remove hypotonic, and add fixative, 2:1 methanol/acetic acid.

(7) Change fixative three times, waiting at least 10 min between changes.

(8) Centrifuge, remove all fixative, and add 1.0 ml 60% glacial acetic acid for each 10 mg of villi. Wait 3–5 min for cells to be released from disrupting villi. Agitate gently to suspend cells.

(9) With half the material, make slides immediately by dropping suspension very gently on cold wet slides. Blow if necessary to improve chromosome spreading and dry on a hot plate.

(10) To the other half of the material, add twice the volume of absolute methanol, slowly, drop by drop, agitating very gently. Drop gently onto cold wet slides.

(11) Heat all slides overnight on hot plate at 45°C, followed by 2 hrs at 65°C the next day. Or just heat 2 hrs at 65°C, then band the same day.

(12) Band with trypsin-Giemsa method. Our modification of the method of Sun et al. [1] is (a) dip slides for 2–7 seconds in coplin jar containing 3–5 ml 10X trypsin-EDTA (Gibco, Grand Island, N.Y.) and 47 ml .025 M potassium phosphate buffer at pH 6.8; (b) rinse in 70% ethanol and then in distilled water; (c) stain on staining rack for 6–8 min using stain mixture made up with 26 ml phosphate buffer, 10 ml methanol, 3 ml 1X trypsin-EDTA and 0.7 ml Giemsa stain. Sometimes the best results are obtained from slides made directly from acetic acid [step (9)] and sometimes from slides made from 2:1 methanol/acetic acid [step (10)].

Culture Method

(1) Use 5–20 mg of clean villi, rinsed twice with 5X PSN (Gibco) in Hank's solution.

(2) Place villi in 35-mm Petri dish; add 0.5–1 ml collagenase solution (3 mg type IV, C-5138, Sigma [St. Louis, Mo.] per ml Hank's solution) and incubate at 37°C for 1 hr.

(3) With a 1.0-ml tuberculin syringe, remove the liquid containing loose cells and place in a sterile 15-ml centrifuge tube in the refrigerator.

(4) Mince the remaining large pieces of villi with small curved scissors, add 1 ml of 1X trypsin-EDTA (Gibco), and incubate at 37°C for 2 hrs.

(5) Break up the cell clumps by aspirating up and down with a 1.0-ml syringe fitted with a 20-gauge needle, then remove the suspension of cells and add to the same tube as in (3).

(6) Add F-10 medium (Gibco) with 20% fetal calf serum to a total volume of 12 ml.

(7) Centrifuge at 800 rpm for 8 min, pour the supernatant into a 25-cm^2 flask, and incubate as a backup culture.

(8) Resuspend the cell button in a total of 2 ml F-10 medium with 20% fetal calf serum and divide into two 35-mm Petri dishes. Add 1 ml more medium to each dish, using Chang's medium if desired.

(9) Cells usually attach in 1 day and can be harvested at 3–7 days, using trypsin-EDTA to remove cells from dish and standard harvesting procedures for fibroblast cultures.

RESULTS

Since this "direct" method allows microscope analysis 48 hrs after biopsy, it is not so rapid as some previously described techniques. However, the yield of metaphases, the length of the chromosomes and the quality of banding obtainable was reproducibly good to excellent in the 20 cases we attempted. From a 10 mg villous sample, we could obtain five to 10 slides, each with five to 20 excellent quality metaphases. Analysis could usually be completed using two to three slides, and extra slides were available for adjusting banding procedures for optimal quality. Banding was routinely analyzable at about the 600-band level. Figure 1 illustrates the quality of the preparations that were regularly obtained from the direct method on villous biopsies.

The culture method allowed harvesting in all 20 cases by at most 7 days. Five cases were ready to harvest in 3 days. The chromosome preparations from these cultured villi were superior or similar in quality to those from the "direct" method: from each Petri dish, five to six slides with 10–20 excellent quality banded metaphases per slide could be obtained.

Using the direct technique, six cases had a 46,XX karyotype, and 14 cases a 46,XY karyotype. Two cases with an XY karyotype on the direct preparation showed 46,XX cells using the culture technique. In one case, only XX cells were seen in 20 cells examined; in the other, one cell of 20 had a 46,XY karyotype. These cultures were harvested 5 and 6 days after initiation. Another case showed mosaicism, in the cultured cells only, for an apparently balanced rearrangement, 46,XY,t(10;11). No 46,XX cells were seen in any of the direct preparations from male cases.

DISCUSSION

The "direct" method described above differs from those previously described by introducing a "holding" period in the cold, with incubation at 37°C

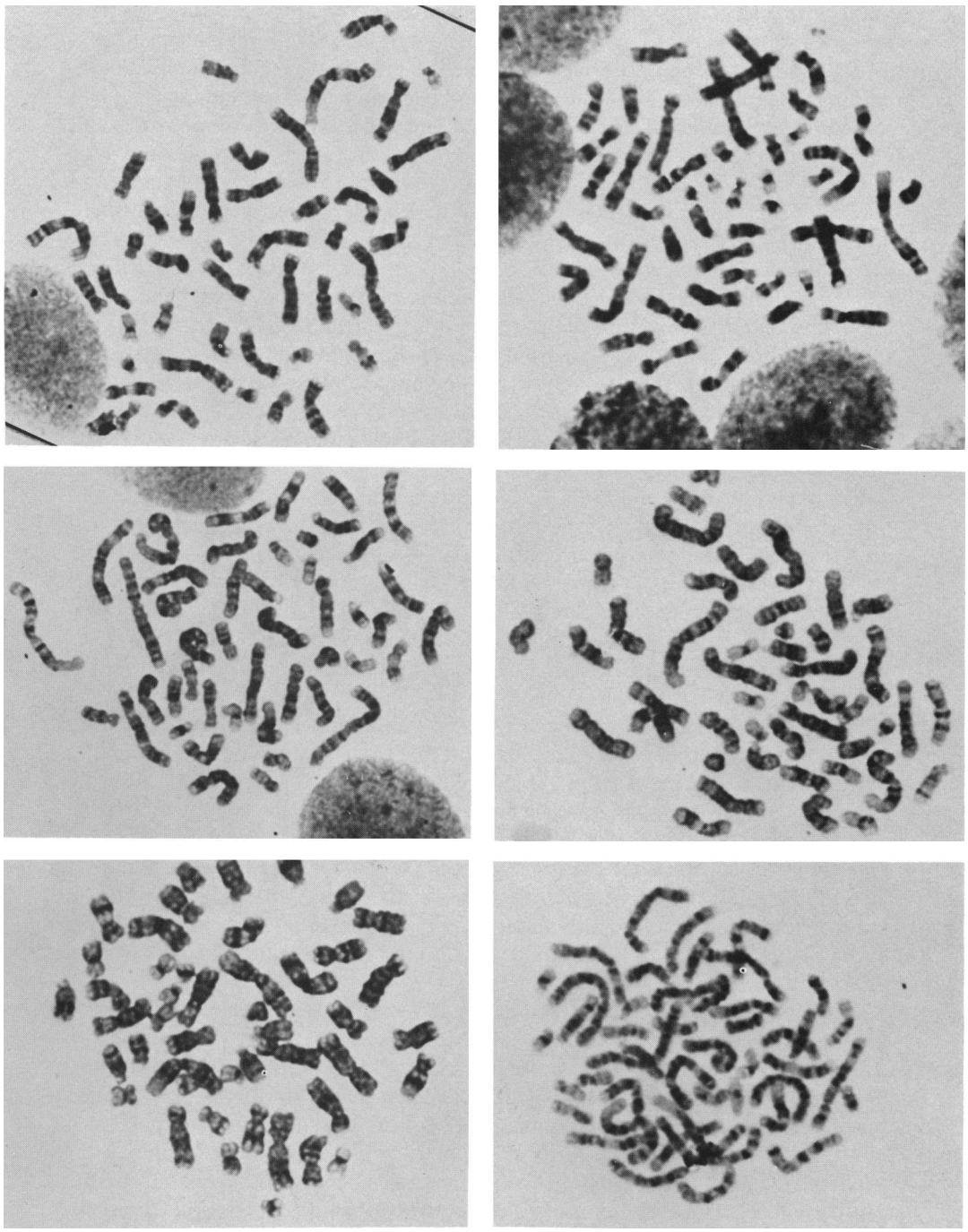

FIG. 1.—Six representative G-banded metaphase figures using the direct method: the two figures *on the top* are from villi obtained from induced abortions of 7 weeks of gestation: the other four figures are from biopsies of chorionic villi at 7–10 weeks.

both before and after this period. It is probable that this cold treatment introduces some degree of synchronization, so that the yield of metaphase figures is higher and Colcemid time can be reduced to half an hour. Other unique parts of our procedure are a 1-hr time of hypotonic treatment with sodium citrate and fixation in 2:1 methanol acetic acid both before and after dissociation of the villi with 60% acetic acid. Slide making uses standard dropping techniques and does not require the special procedures described by Simoni et al. [2] or Gregson and Seabright [3]. Specimens received late in the day can be refrigerated overnight and the whole procedure begun the next morning.

In our last 20 biopsies, we have been able to analyze 20 well-banded cells (at approximately the 600-band stage) from direct preparations whose quality (see fig. 1) and quantity was reproducibly superior to those we have seen with other published direct methods. Using these other direct methods, we were seldom able to obtain more than 10 analyzable metaphase plates from one specimen and the chromosomes were usually short and poorly banded. Complete analysis (20 cells, some at 600-band stage) was only possible by also using material from cultured cells.

Although our direct method does not provide results in 24 hrs, it does avoid breaking up of the villi prior to harvesting, with subsequent attachment and growth of cells in a monolayer culture. This is important because such culture can apparently lead to overgrowth by the small amounts of maternal decidual tissue that may remain even after careful dissection of villi. In the last 20 cases where we have used the techniques described above, we have had two cases where this occurred, out of a total of 14 male fetuses. We have to assume that maternal contamination occurs as often in female cases but cannot be recognized by karyotyping alone. In four cases in our laboratory, we have compared the growth in culture of decidual cells with that of disrupted villi and found that the decidual tissue grew more rapidly. This behavior of decidual tissue from normal healthy pregnancies is in contrast to decidual tissue obtained from spontaneous abortion specimens, which rarely grows in culture, presumably because it is necrotic after the normal spontaneous abortion process. We have also deliberately mixed decidual and villous tissue in two cases with an XY karyotype and did not find any maternal cells in our direct preparations. However, Blakemore et al. [4] did find a small number of decidual metaphases on direct preparation.

The rate of maternal cell growth in cultured tissues in our hands is presently too high to be clinically acceptable as the sole procedure. More experience with dissecting out decidual tissue or better biopsy specimens might mitigate this problem. However, discussion with colleagues leads us to believe that the problem of maternal cell contamination of cultured tissues is not unique to our laboratory: it may be more serious in laboratories that are just beginning the procedures and are inexperienced in sampling and dissecting villous tissue. Methods that can avoid culture and still reliably provide chromosome preparations of the same or nearly the same quality may thus be preferable to culture methods, since the evidence suggests that they are unlikely to yield misleading results due to maternal contamination. However, culture must be provided as a

backup in case the direct method should fail or the initial analysis suggests chromosomal problems that require further cytogenetic analysis.

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