groups, systemic and local reactions were reported consistently less often in the accelerated schedule group. This suggests that the observed differences were due to the age difference between infants receiving each schedule.

Our study suggests that an accelerated schedule of immunisation is likely to cause fewer reactions than the prolonged schedule. We advise the continued close monitoring of vaccine coverage and adverse event reports after implementing accelerated immunisation.

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New rapid test for prenatal detection of trisomy 21 (Down's syndrome): preliminary report

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Abstract

Objective—To devise and evaluate a rapid screening method for detecting trisomy 21 (Down's syndrome) in samples of uncultured amniotic fluid cells.

Design—Non-radioactive in situ hybridisation with HY128, a 500 000 base pair yeast artificial chromosome probe specific for chromosome 21. Blinded study of 12 karyotypically normal amniotic fluid samples and eight samples trisomic for chromosome 21.

Setting—Cytogenetic and obstetric services at a tertiary referral centre, Copenhagen.

Main outcome measures—Time necessary to complete the test. Proportion of cell nuclei containing two and three hybridisation signals in karyotypically normal and abnormal amniotic fluid samples.

Results—The test could be completed within three to four days after amniocentesis. In the normal samples a mean of 73% (range 61-82%) of the amniotic cell nuclei showed two hybridisation signals and 6% (0-18%) showed three signals. By contrast, among the trisomic samples 29% (19-38%) of the nuclei exhibited two signals and 48% (31-60%) showed three signals.

Conclusion—The technique clearly distinguished between normal and trisomic samples. Prenatal diagnosis with in situ hybridisation with chromosome specific probes was fast and may make it possible to screen for selected, aneuploidies. However, the technique is still at a preliminary stage and needs further evaluation and refinement.

Introduction

Non-radioactive in situ hybridisation has made it feasible to obtain cytogenetic information from interphase cells. In this technique chemically modified probe DNA, specific for a certain chromosome, is mixed with chromosomal DNA after denaturation to separate the double strands. After the probe and chromosomal DNA have combined the probe is conjugated with fluorescent molecules which can then be visualised by fluorescence microscopy.

Several studies have shown that non-radioactive in situ hybridisation with DNA probes can be used to study aberrations in the number of copies of chromosomes in cultured amniocytes.¹⁻⁴ We have previously

reported the use of two centromere specific probes to determine the number of copies of chromosomes 1 and 18 in samples of uncultured amniocytes.⁴ Another study has described enumeration of the Y chromosome in uncultured amniocytes by in situ hybridisation with a probe specific for the long arm of this chromosome.⁵ Similarly, we have found the centromere specific, chromosome X specific probe pBamX⁶ and a probe specific for a locus on the long arm of chromosome 13^{7,8} are suitable for in situ hybridisation on uncultured amniocytes (unpublished data).

In this preliminary study we used in situ hybridisation with a large probe specific for chromosome 21 to try to distinguish between samples of uncultured amniocytes with normal and trisomic karyotypes.

Materials and methods

CELLS

Amniotic fluids were sampled at 14-21 weeks' gestation from women at moderate risk of a fetus with chromosome abnormalities. Women carrying fetuses with karyotypically confirmed Down's syndrome had amniotic fluid drawn in conjunction with termination of pregnancy at 13-22 weeks' gestation. Informed consent was given by all the women. Amniocytes were isolated and prepared for in situ hybridisation as previously described.⁴ Twelve samples from fetuses with a normal karyotype and eight samples from fetuses with trisomy 21 were further analysed as described below.

Cultured amniocytes and mesenchymal cells from the chorionic villi were prepared by standard procedures and used for interphase and metaphase studies.

PROBES

The yeast artificial chromosome HY128 contains a human chromosome 21 specific DNA fragment of about 500 000 base pairs. HY128 was obtained from M-C Potier and M Goedert (Medical Research Council, Cambridge, United Kingdom).

DNA from the HY128 yeast artificial chromosome and the host was used as the chromosome 21 probe and was prepared essentially as described by Burke and Olson.⁹ The probe was labelled with biotin-14-dATP (Bethesda Research Laboratories, Gaithersburg, Maryland, USA).

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IN SITU HYBRIDISATION

In situ hybridisation with HY128 was done by using a modification of the procedure described by Pinkel *et al.*¹⁰ Target DNA on slide mounted cells was denatured in 60% formamide, twice strength SSC buffer (normal strength SSC=0.15 M sodium chloride, 15 mM sodium citrate; pH 7.0) for 7 minutes at 80°C. Probe DNA was mixed 1:11 (w/w) with sonicated human placental DNA and desiccated under vacuum. The DNA was resuspended to a final concentration of 50 ng/μl in a hybridisation mixture consisting of 50% formamide, twice strength SSPE buffer (normal strength SSPE=0.15 M sodium chloride, 10 mM sodium dehydrogen phosphate, 1 mM EDTA; pH 7.4), and 10% dextran sulphate. This solution was denatured at 70°C for 5 minutes and applied to cells on dry slides (3.2 μl/cm²). A coverslip was overlaid and sealed with rubber cement. Hybridisation was allowed to continue at 37°C in a humidified chamber for 36-60 hours. Slides were then washed in three changes of 50% formamide, twice strength SSC buffer at 45°C for 2 minutes each, twice in twice strength SSC buffer at room temperature for 5 minutes each, and once in

quadruple strength SSC buffer,¹¹ 0.05% Tween 20 at room temperature for 5 minutes. Slides were then treated with alternating layers of fluoresceinated avidin and biotinylated goat antiavidin both at 5 μg/ml in quadruple strength SSC buffer, 5% non-fat dry milk for 20 minutes each until two layers of avidin were applied. The avidin and antiavidin treatments were separated by three washes of 5 minutes each in quadruple strength SSC buffer, 0.05% Tween 20 (avidin-FITC and biotinylated antiavidin from Vector Laboratories, Burlingame, California, USA).

Slides were mounted in Tris glycerol buffer containing an antifading agent (1,4-diazabicyclo-(2,2,2)-octane (DABCO, Sigma, St Louis, Missouri, USA). The mounting medium contained either a blue (4',6-diamidino-2-phenylindole dihydrochloride (DAPI, Boehringer Mannheim, Penzberg, Germany) or a red DNA counterstain (propidium iodide).

Slides were examined with a Leitz Diaplan microscope equipped for fluorescein, DAPI, and propidium iodide fluorescence. Photomicrographs were all taken at 2800 times magnification on Kodak Ektar 1000 film.

BLIND STUDY DESIGN

All slides were evaluated quantitatively by the same blinded person. Because the number of squamous cells in amniotic fluid varies with gestational age¹² the normal and trisomic samples were collected at about the same gestational age range.

One slide per patient was examined. If possible, about 100 nuclei per slide were counted. Squamous cells, nuclei without signals, overlapping nuclei, and nuclei covered by cytoplasm were not scored. Patchy and diffuse signals were included in the evaluation only if they were well separated. Split spots—that is, signals in a paired arrangement—were scored as one signal if each signal was weaker than other signals in the same nucleus or as two signals if the intensities of the two spots were alike and equal to or stronger than the intensities of other signals in the same nucleus.¹³

Results

CULTURED CELLS¹⁴

Metaphase spreads and interphase nuclei of cultured amniocytes and mesenchymal cells of chorionic villi were used to optimise the in situ hybridisation assay. Hybridisation with HY128 resulted in discrete signals on both q arms of a pair of G group chromosomes in metaphase spreads from cells with a normal karyotype. Under the described stringency conditions for hybridisation and for posthybridisation washes, no signals on other chromosomes were detected. Hybridisations to cells with a 47,+21 karyotype confirmed the specificity of HY128 hybridisation to chromosome 21 (fig (a) and (b)).

BLINDED STUDY ON UNCULTURED AMNIOCYTES

Although the signals from HY128 in situ hybridisation were generally smaller and harder to detect in uncultured than cultured amniocytes (fig (c) and (d)), signals were scorable.

The table summarises the results of the blinded study. In samples of normal karyotype an average of 73% (range 61-82%) of the scored nuclei on a slide showed two signals while 6% (0-18%) of the nuclei had three hybridisation signals. By contrast in samples trisomic for chromosome 21 an average of 48% (31-60%) of the amniocyte nuclei showed three signals, while 29% (19-38%) had two signals. About the same fraction of nuclei in both disomic and trisomic sample sets (20%) showed a single signal, while about 3% of nuclei showed four signals. Perhaps more interesting is the ratio of nuclei showing three and two signals (table). For the normal samples this ratio averaged

Photomicrographs showing in situ hybridisation of HY128 probe to cell nuclei without surrounding cytoplasm. (a) and (b), Hybridisation to metaphase and interphase preparations of cultured, mesenchymal, chorionic villi cells with 47,+21 karyotype. Three G group chromosomes all exhibit two signals, one for each chromatid. In interphases the two signals at each chromosome fuse to one signal (propidium iodide counterstain). (c) Hybridisation to uncultured amniocytes with normal karyotype. (d) Hybridisation to uncultured amniocytes with 47,+21 karyotype (counterstained with DAPI, which is not excited at same wavelength of fluorescein)

Karyotype	Average % (range) of nuclei showing:				
	1 signal	2 signals	3 signals	4 signals	3:2 ratio*
Normal (n=12)	20 (10 to 36)	73 (61 to 82)	6 (0 to 18)	2 (0 to 5)	0.08 (0.00 to 0.29)
47,+21 (n=8)	19 (7 to 31)	29 (19 to 38)	48 (31 to 60)	4 (0 to 9)	1.76 (0.83 to 3.08)

*Proportion of nuclei with three signals divided by proportion with two signals.

0.08, range (0.00-0.29) while in the trisomy 21 samples it averaged 1.76 (0.83-3.08). In only one of the trisomy samples was this ratio less than 1.

The average number of amniocyte nuclei scored for each sample was 83 (34-114).

Discussion

In this preliminary study we found that by using a large locus specific probe we could count copies of chromosome 21 by in situ hybridisation in samples of uncultured amniocytes three to four days after amniocentesis. In comparison conventional chromosome analysis takes one and a half to three weeks.

Identification of normal and abnormal samples by fluorescence in situ hybridisation was not simple. Some cells in samples from fetuses with Down's syndrome had two chromosome 21 signals and some cells in samples from normal fetuses had three signals. Sample quality, probe characteristics, and hybridisation conditions affect the hybridisation efficiency and specificity, which in turn affect scorability of the sample. The absence of expected signals may be explained by loss of cellular target DNA or failure of the probe to bind or fluoresce. Extra signals (including split spots) may be caused by large chromatid separation, DNA replication, or cross hybridisation of the probe to other targets.

Hybridisation artefacts (for example, fluorescing debris on the slide, prevention of probe access to the target DNA by small air bubbles) may contribute to both types of unexpected hybridisation patterns. Improvements in hybridisation procedures and sample preparation have been shown recently,¹⁵ indicating that techniques can be further optimised for greater hybridisation efficiency and signal discrimination.

Correct identification of fetuses with Down's syndrome was possible because the ranges of proportions of nuclei showing two or three signals in normal and abnormal samples did not overlap (table). The ratio of three to two signals was distinct in normal and trisomic samples. Because the proportions of nuclei having one and four signals were fairly constant regardless of karyotype the 3:2 ratio offers a logical means of discrimination between normal and aberrant samples.

REPRODUCIBILITY OF RESULTS

We have found that interobserver scoring variability is minimal at our laboratory.⁴ However the 3:2 ratio may vary from one laboratory to another because of the subjective scoring system. The scoring system was designed to address the occasional presence of split spots, attributed to DNA replication before cell division.¹⁶ The subjectivity of the system may make scoring consistency difficult. In recent clinical studies using fluorescent hybridisation probes on uncultured amniocytes, analysis of data derived from a direct scoring protocol showed that the trisomic group did not overlap with the normal group (K W Klinger *et al*, personal communication). These results imply that the presence of split spots did not significantly affect the distinction between normal and trisomic samples. In future clinical studies direct counting criteria will be adopted to diminish scoring subjectivity.

This preliminary study was designed to determine

the feasibility of detecting trisomy 21 in samples of uncultured amniocytes with a chromosome 21 specific probe. The analyses were done in conjunction with conventional chromosome analyses. The numbers of samples scored in this and our previous study⁴ were too small to define the criteria for detecting aneuploidy. Different detection criteria may be required for each probe since there may be considerable variation in signal size between probes. Because the prevalence of trisomies in this small sample was high it is difficult to predict the accuracy of the test when sampling from a population with a lower prevalence of trisomy 21.

VALUE OF NON-RADIOACTIVE IN SITU HYBRIDISATION

Non-radioactive in situ hybridisation may become increasingly used in prenatal diagnosis. With the addition of the probe specific for chromosome 21 it is now possible to detect numerical abnormalities of chromosomes 21, 18,⁴ 13, X, and Y³ in samples of uncultured amniocytes. Because the probes are site specific and cover at most 10 000 000 base pairs⁶ rearrangements such as inversions, translocations, and ring chromosomes will usually be missed, as will abnormalities of chromosomes other than 21, 18, 13, X, and Y. Nevertheless, about 95% of the clinically significant cytogenetic disorders should be detected by performing in situ hybridisation with a set of site specific probes hybridising to these five chromosomes.¹⁷

In any prenatal test total accuracy is essential because pregnancy may be terminated on the basis of the result. Conventional chromosome analysis has been established as the reliable test for chromosomal abnormalities, although the cell culture is expensive and results take up to three weeks to obtain. In future, detection of aneuploidies by in situ hybridisation may be of value as a rapid means of screening samples, augmented with conventional chromosome analysis on samples with positive or indeterminate results. Large, prospective clinical trials will have to be undertaken to establish the proper diagnostic protocols and criteria. In these trials the prevalence of trisomic samples should match the incidence of Down's syndrome in future test populations. We have begun a prospective study of amniocyte samples, incorporating improvements in in situ hybridisation technology with these aims.

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Detection of IgA and IgM antibodies to HIV-1 in neonates by radioimmune western blotting

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Abstract

Objective—To detect infection with HIV-1 by IgA and IgM response at birth in children born to HIV-1 seropositive mothers.

Design—Western blotting and radioimmune western blotting on stored sera from infected and uninfected babies born to HIV-1 seropositive mothers. Sera were pretreated to remove IgG.

Setting—Parma and Bologna, Italy.

Subjects—12 infected and five uninfected babies born to HIV-1 seropositive mothers and three babies born to seronegative mothers.

Main outcome measures—Effectiveness of western blotting and radioimmune western blotting in detecting antibodies to HIV-1 gene products.

Results—With conventional western blotting we found IgA class antibodies to HIV-1 proteins in serum from three out of 12 infected children; in two of these three the serum was collected at age 3 months (positive controls). Radioimmune western blotting detected both IgA and IgM antibodies in serum from all infected children tested, whereas all serum from uninfected children born to seropositive and seronegative mothers showed no such antibodies.

Conclusion—Although the technique should be tested on more patients, radioimmune western blotting seems to be a valuable tool for serological diagnosis of congenital HIV-1 infection at birth in neonates born to seropositive mothers.

Introduction

By the end of this century AIDS may be the main cause of death among children.¹ Around 15-30% of babies born to seropositive mothers are infected with HIV, and among these 83% will show clinical or laboratory signs by the age of 6 months.^{2,3}

Early diagnosis of HIV-1 infection is an essential requisite to intervention with antiviral or other treatment because the clinical symptoms in infected children often appear late in the course of infection. Many methods have thus been used to define an infected state. Viral culture has proved sensitive, but it requires one to four weeks to become positive, necessitates special biosafety precautions, is costly and labour intensive,^{4,5} and not all infected children give positive cultures at birth.^{6,7}

The p24 antigen assay is of limited use because of the presence of immune complexes which may mask the HIV-1 antigen.^{8,9} The "in vitro antibodies production assay"¹⁰⁻¹² and the related Elispot technique¹³ detect lymphocytes producing HIV-1 antibodies, but this method is only effective after the first month of life.

The polymerase chain reaction is the only technique

that permits a rapid and low cost diagnosis within the first two months of life and requires only 1-3 ml of blood,^{8,9,14,15} but the results are not always reliable because of false positive results.¹⁶

Serological tests form the basis for the diagnosis of HIV infection in adults, but in children born to seropositive mothers they are hampered by the presence of passively transmitted IgG antibodies, which may be detectable up to 18 months after birth.^{2,8,17}

Since IgA and IgM antibodies do not cross the placenta they are a reliable sign of infection but their small amount as well as the transient production of IgM antibodies and the presence of maternal IgG may render enzyme linked immunosorbent assays (ELISA) and western blot assays unreliable. Removal of IgG with recombinant protein G allows detection of specific IgA in most infected children aged at least 12 months; in younger children, however, the results are less reliable and the positive results of IgM detection are reduced by up to half with IgA antibodies,^{18,19} probably because of the low sensitivity of the method used.

We thus developed a more sensitive method to detect antibodies to HIV-1 proteins; it consists of a modified western blot procedure (radioimmune western blotting) in which biotinylated antibodies to HIV-1 antibodies are detected by means of isotope (sulphur-35 or iodine-125) labelled streptavidin.²⁰ We used both western blotting and radioimmune western blotting on stored serum from infected (G Furlini, *et al*, seventh international conference on AIDS, Florence, 1991; abstract No WA 1340) and uninfected children born to seropositive mothers as well as on serum from seronegative children born to mothers without any history of HIV-1 infection to search for HIV-1 specific IgA and IgM antibodies.

Subjects and methods

SUBJECTS

This unblinded survey was performed on 70 children born to seropositive mothers in Bologna. Infection had been verified in only 12 (17%); the remaining 58 (83%) were uninfected as determined by viral culture or p24 antigen assay, or both. For 10 out of the 12 infected children the serum was collected at birth. In the remaining two serum was collected aged 3 months to guarantee the presence of IgA or IgM antibodies to HIV-1 protein, or both, and represented our positive controls for infected children in radioimmune western blotting. Five of the 58 uninfected children were randomly selected as negative controls for uninfected children born to seropositive mothers. Serum from three children born to seronegative mothers were tested as further controls. All serum

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