ANALYSIS OF AMNIOTIC FLUID CELLS FOR DIAGNOSIS OF FOETAL SEX

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For many years attempts have been made, by different approaches, to develop a method that would give a reliable prenatal diagnosis of foetal sex. We have previously shown (Serr, Sachs, and Danon, 1955; Sachs, Serr, and Danon, 1956) that a reliable prenatal diagnosis can be made by a study of chromocentres in amniotic fluid cells.

This method of diagnosing sex is based on a well-established theoretical and practical foundation, since it is known that in humans, as in most other mammals, there is a difference in the sex chromosome constitution of normal males and females—males having the sex chromosome constitution XY, and females the sex chromosome constitution XX (reference in Sachs, 1954, 1955)—and since identification of normal males and females by a study of chromocentres has invariably given correct results when applied to human cells from other sources (Moore, Graham, and Barr, 1953; Moore and Barr, 1954, 1955; Emery and McMillan, 1954; Marberger and Nelson, 1955; Marberger, Boccabella, and Nelson, 1955; *Lancet*, 1955; Sachs and Danon, 1956).

The present paper deals with our detailed findings in an analysis of amniotic fluid cells in 40 cases in the ninth month, and in 8 cases in the third, sixth, and seventh months of pregnancy.

Material and Methods

Amniotic fluid was obtained prior to delivery by puncture of the membranes, and in the earlier months of pregnancy, excepting the case in the third month, by abdominal puncture (Dieckmann and Davis, 1953; Alvarez and Caldeyro, 1950). The case in the third month was obtained during hysterectomy performed by Professor B. Zondek.

The fluid was centrifuged at 2,500 rotations a minute for five minutes. The sediment, which contained the cells, was smeared on slides that had previously been coated with Mayer's albumin, and then fixed for at least one hour in a fixative consisting of equal parts of absolute alcohol and ether. We also tested 3:1 alcohol-acetic acid, and a modified Kahle's fixative (see below), and although these were both satisfactory they were not superior to the alcohol-ether, which was therefore routinely used. If the slides had to be kept for any length of time after fixation, or if they had to be transported, they were rinsed in 70% alcohol, 50% alcohol, water, and then kept after rinsing in glycerin-water.

For the study of chromocentres we routinely used Feulgen staining, with fast green as a counterstain for the cytoplasm. The Feulgen method of staining is specific for desoxyribose

nucleic acid, a chemical constituent that is found only in the chromosomes, and it therefore gives a clear staining of the chromocentres.

For the Feulgen staining, the slides, after rinsing in water, were transferred for eight minutes to normal hydrochloric acid at 60° C., stained with leucobasic fuchsin for one hour, and this was followed by three rinses, totalling 10 minutes, in SO₂ water, and three rinses in distilled water. The slides were then rinsed in 70% alcohol, 95% alcohol, counterstained in a dilute fast green solution in 95% alcohol, followed by two rinses in 95% alcohol, and a rinse in absolute alcohol, equal parts of absolute alcohol and xylol, xylol, and mounting in canada balsam.

For the analysis of the different types of cells the slides were stained, after alcohol-ether fixation, with Papanicolaou EA-36 and OG-6.

When sections of embryonic and foetal membranes were examined, pieces were fixed in a modified Kahle's fixative consisting of 100 parts absolute alcohol, 7 parts glacial acetic acid, and 40 parts 40% formaldehyde. After fixation for at least 12 hours, the pieces were transferred to 70% alcohol if they had to be stored. For embedding they were passed through 95% alcohol, absolute alcohol, equal parts

FIG. 1.—Photomicrographs of cells from the amniotic fluid and the skin of human foetuses. No. 13 stained with Papanicolaou; all others stained with Feulgen and fast green. 1, 2, and 3, Cells from the amniotic fluid of 9-months-old female foetuses. Chromocentre at the nuclear membrane. ($\times 1,500$.) 4, Cell from the amniotic fluid of a 6-months-old female foetus. Chromocentre at nuclear membrane. ($\times 1,500$.) 5 and 6, Cells from the amniotic fluid of 9-months-old male foetuses. Chromocentre absent. ($\times 1,500$.) 7 and 8, Cells from the amniotic fluid of 5-months-old male foetuses. Chromocentre absent. ($\times 1,500$.) 9, Piece of tissue with 70 diagnosable cells from the amniotic fluid of a 5-months-old male foetus. Chromocentre absent. ($\times 500$.) 10 and 11, Sections of skin from 6-months-old male foetuses. Chromocentre absent. ($\times 760$.) 12 and 13, General view of smears from the amniotic fluid of 9-months-old foetuses. ($\times 130$.)

TABLE I.—Percentage of Cells in Amniotic Fluid Suitable for Diagnosis of Sex. In Each Case there are 5 Separate Counts of 100 Cells

Males	Case No. . .	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
	Cells %	13 5 7 9 8	13 13 15 20 9	8 11 11 10 11	14 16 13 14 9	8 8 6 10 9	11 7 11 15 12	10 6 18 8 10	12 15 17 16 12	7 9 13 12 11	14 16 18 13 11	9 13 13 16 9	20 17 11 12 15	6 13 9 12 10	17 14 12 14 11	23 17 10 19 12
	Mean . . . Standard error ±	8.4 1.3	14.0 1.8	10.2 0.6	13.2 1.2	8.2 0.7	11.2 1.3	10.4 2.0	14.4 1.0	10.6 1.1	14.4 1.2	12.0 1.3	15.0 1.6	10.0 1.2	13.6 1.0	16.2 2.4
Females	Case No. . .	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
	Cells %	13 11 8 11 7	15 9 12 14 12	18 10 16 11 10	12 10 16 10 11	18 16 16 13 11	11 6 3 10 7	8 7 11 9 10	21 14 19 13 16	9 14 10 7 8	19 9 14 19 17	21 17 17 20 21	19 14 17 13 19	18 15 16 10 13	11 12 11 11 11	8 7 10 10 8
	Mean . . . Standard error ±	10.0 1.1	12.4 1.0	13.0 1.7	11.0 0.4	14.8 1.2	7.4 1.4	9.0 0.7	16.6 1.5	9.6 1.2	15.6 1.9	19.2 0.7	16.4 1.3	14.4 1.4	11.2 0.2	8.6 0.6

of absolute alcohol and xylol, two changes of xylol, and embedded in wax. Sections were cut at 10 μ , and the sections, like the smears, were stained with Feulgen and fast green for the study of chromocentres, and with Papanicolaou for the cell types.

Results

Types of Cells in the Amniotic Fluid

An analysis of the types of cells in the amniotic fluid was made on Papanicolaou smears of fluid taken prior to delivery. Three main types of epithelial cells were found, which could be classified (Vincent Memorial Hospital, 1950) as basal cells with vesicular nuclei and green-staining cytoplasm; precornified and cornified cells with vesicular to pyknotic nuclei and square or folded and wrinkled cytoplasm which stains green in the precornified and pink in the cornified cells; and keratinized cells with advanced nuclear degeneration or no nucleus at all and an orange-staining wrinkled cytoplasm. In many anucleate cells the cytoplasm was not stainable.

The basal and precornified cells, with green-staining cytoplasm, are the most suitable for the sex diagnosis. Most of the cells with pink-staining cytoplasm and the cells with orange-staining cytoplasm are unsuitable since they have nuclei with different kinds of degeneration such as pyknosis, loss of stainability, and karyorrhexis.

In order to determine the origin of the cells suitable for the diagnosis of sex we examined Papanicolaou-stained sections of skin from a 6-months-old foetus, and Papanicolaou-stained sections of skin and smears of the oral mucosa and vagina from a newborn infant. The skin of the 6-months-old foetus was found to have a surface sheet of cornified cells without nuclei and pink- or orange-staining cytoplasm. In some places this sheet of dead cells had peeled off, so that small patches of cells suitable for diagnosis could then be in contact with the amniotic fluid. In the skin of the newborn infant there was a continuous surface sheet of dead cells in the pieces that were examined, whereas both the oral mucosa and the vaginal smears showed that nearly all the cells of the surface layers were suitable for diagnosis. The cornified and keratinized cells found in the amniotic fluid thus seem to come from the skin, and it can be assumed that the cells suitable for diagnosis come to a large extent from the mucosae of the foetus. The epithelium of the amniotic membrane as a possible source of suitable cells is also not excluded.

A small proportion of polymorphonuclear leucocytes were found in the amniotic fluid. But these were all degenerate or badly spread, so that it was not possible to make a sex diagnosis such as that which can be made on these cells in blood smears taken after birth (Davidson and Robertson Smith, 1954; Sachs and Danon, 1956).

Percentage of Cells Suitable for the Diagnosis of Sex

Since the amniotic fluid contains many cells that are not suitable for sex diagnosis, we determined the percentage of

diagnosable cells in cases prior to delivery and in earlier stages of pregnancy. The results of five separate determinations on 100 cells each in 15 males and 15 females are given in Table I. In both males and females, Cases 1 and 12 are prior to delivery. In the males, Cases 13 and 14 are from a 5½-months-old foetus and Case 15 from a 6-months-old foetus; and in the females, Case 13 is from a 5½-months-old foetus and Cases 14 and 15 from a 6-months-old foetus.

It can be seen from Table I that the percentages of diagnosable cells in the five- and six-months cases are similar to those found in some of the later cases. One case of hydramnios with a male foetus that was examined in the middle of the ninth month gave, on a count of 500 cells, an average of $15.2 \pm 1.2\%$ diagnosable cells, and this is equivalent to Case 12 among the normal cases of pregnancy. In one of the sixth-months cases there was a piece of tissue in the amniotic fluid with 70 diagnosable cells (Fig. 1, No. 9). A comparison of the totals of the 15 cases in males and females gives an average of 12.1 ± 0.2 for males and 12.4 ± 0.9 for females, so that there is no significant difference between the two sexes.

In the one case that was at the end of the third month there was a total of 36 cells (in 4 ml. of amniotic fluid), of which 22 (61%) were suitable for diagnosis.

Study of Chromocentres for Diagnosis of Sex

The diagnosis of sex on the amniotic fluid cells is based on determinations of the percentage of cells with a chromocentre that may or may not be at the nuclear membrane. The results of five separate counts of 100 cells each in five males and five females prior to delivery are shown in Tables II and III.

The data on 40 cases in the ninth month (Table IV, Figs. 2 and 3), which consist of 39 cases prior to delivery and 1 case of hydramnios in the middle of the ninth month, show that there is a clear difference both in the percentages of cells with a chromocentre and in the percentage of cells with a chromocentre at the nuclear membrane, in the cases diagnosed as males and females. In each of these cases the diagnosis of sex on the amniotic fluid cells was confirmed at delivery.

In the seven cases in the sixth and seventh months (Table V), there is also a clear difference between males and females. One of these was a pathological pregnancy in a woman who had been bleeding intermittently for two months before she was examined, and the diagnosis of sex on the amniotic fluid cells was confirmed both from the appearance of the foetus and from an analysis of a skin biopsy. The other six cases have not yet been delivered, but since there were a sufficient number of diagnosable cells there is no reason to doubt that, as in all the other cases, the diagnosis will be confirmed at delivery.*

*The sex of these foetuses has now been confirmed at delivery.

TABLE II.—Percentage of Cells with a Chromocentre, in the Amniotic Fluid of Male Foetuses. In Each Case there are 5 Separate Counts of 100 Cells

	% Cells With Chromocentre			% Cells Without Chromocentre
	At Nuclear Membrane	Not at Nuclear Membrane	Total	
Case 1	9	11	20	80
	6	6	12	88
	7	9	16	84
	5	7	12	88
	12	8	20	80
Mean	7.8	8.2	16.0	84.0
Standard error	1.2	0.9	1.8	1.8
Case 2	5	22	27	73
	3	13	16	84
	7	9	16	84
	5	10	15	85
	12	11	23	77
Mean	6.4	13.0	19.4	80.6
Standard Error	1.5	2.3	2.4	2.4
Case 3	4	7	11	89
	1	6	7	93
	5	8	13	87
	3	3	6	94
	5	6	11	89
Mean	3.6	6.0	9.6	90.4
Standard error	0.7	0.8	1.1	1.1
Case 4	4	7	11	89
	8	8	16	84
	4	11	15	85
	9	8	17	83
	9	10	19	81
Mean	6.8	8.8	15.6	84.4
Standard error	1.2	0.7	1.3	1.3
Case 5	8	1	9	91
	6	11	17	83
	3	7	10	90
	4	3	7	93
	6	8	14	86
Mean	5.4	6.0	11.4	88.6
Standard error	0.9	1.8	1.8	1.8

TABLE III.—Percentage of Cells with a Chromocentre, in the Amniotic Fluid of Female Foetuses. In Each Case there are 5 Separate Counts of 100 Cells

	% Cells With Chromocentre			% Cells Without Chromocentre
	At Nuclear Membrane	Not at Nuclear Membrane	Total	
Case 1	25	28	53	47
	33	13	46	54
	30	21	51	49
	35	17	52	48
	24	18	42	58
Mean	29.4	19.4	48.8	51.2
Standard error	2.2	2.5	2.1	2.1
Case 2	48	23	71	29
	38	19	57	43
	38	37	75	25
	43	26	69	31
	37	21	58	42
Mean	40.8	25.2	66.0	34.0
Standard error	2.1	3.2	3.6	3.6
Case 3	29	26	55	45
	29	29	58	42
	29	17	46	54
	27	24	51	49
	28	28	56	44
Mean	28.4	24.8	53.2	46.8
Standard error	0.4	2.1	2.1	2.1
Case 4	38	19	57	43
	42	20	62	38
	42	18	60	40
	46	24	70	30
	41	26	67	33
Mean	41.8	21.4	63.2	36.8
Standard error	1.3	1.5	2.4	2.4
Case 5	35	27	62	38
	42	19	61	39
	46	17	63	37
	39	18	57	43
	47	23	70	30
Mean	41.8	20.8	62.6	37.4
Standard error	2.2	1.9	2.1	2.1

TABLE IV.—Percentage of Cells with a Chromocentre, in the Amniotic Fluid in 40 Cases in the Ninth Month of Pregnancy. Figures in Each Case are Based on a Count of 200 or 500 Cells

	Males			Females		
	% Cells with Chromocentre			% Cells with Chromocentre		
	At Nuclear Membrane	Not at Nuclear Membrane	Total	At Nuclear Membrane	Not at Nuclear Membrane	Total
0.5	0.5	6.0	6.5	22.0	12.0	34.0
1.0	1.0	6.5	7.5	26.5	15.5	42.0
1.5	1.5	11.0	12.5	28.0	11.5	39.5
2.0	2.0	6.5	8.5	29.0	27.5	56.5
2.5	2.5	6.0	8.5	29.4	19.4	48.8
3.0	3.0	8.0	11.0	32.0	14.5	46.5
3.0	3.0	11.0	14.0	32.5	27.0	59.5
3.0	3.0	11.5	14.5	34.0	10.0	44.0
3.5	3.5	4.5	8.0	34.0	11.5	45.5
3.5	3.5	8.0	11.5	34.0	12.5	46.5
3.5	3.5	10.5	14.0	34.0	31.0	65.0
3.5	3.5	11.5	15.0	34.5	11.0	45.5
3.6	3.6	6.0	9.6	35.0	11.5	46.5
5.4	5.4	6.0	11.4	37.0	21.5	58.5
5.5	5.5	5.5	11.0	38.0	17.0	55.0
6.4	6.4	13.0	19.4	40.0	16.5	56.5
6.8	6.8	8.8	15.6	40.0	19.0	59.0
7.8	7.8	8.2	16.0	40.8	25.2	66.0
				41.8	20.8	62.6
				41.8	21.4	63.2
				43.5	15.5	59.0
				44.5	20.0	64.5
Mean	3.7	8.3	11.9	35.1	17.8	52.9
Standard error	0.5	0.2	0.8	1.3	1.3	2.0

FIG. 2.—Histogram of the percentage of cells with a chromocentre, in amniotic fluid cells from 40 cases in the ninth month of pregnancy. The data have been grouped in intervals of 5.

FIG. 3.—Histogram of the percentage of cells with a chromocentre at the nuclear membrane, in amniotic fluid cells from 40 cases in the ninth month of pregnancy. The data have been grouped in intervals of 5.

In the one case that was obtained during hysterectomy at the end of the third month, out of the 22 cells suitable for diagnosis in 4 ml. of fluid there were 21 with a chromocentre, in 12 of which the chromocentre was at the nuclear membrane. The diagnosis of female sex on the amniotic fluid cells was confirmed by a study of embryonic membranes. We also found it possible to make a diagnosis on the cells in the fluid of an 8-weeks-old aborted embryo, and this was again confirmed by a study of embryonic membranes.

In some cases prior to delivery, but never in any of the earlier cases, the fluid was obviously contaminated with cells from the mother, and if there was a male foetus it was possible to distinguish both male and female patches of cells. In these contaminated cases, which have of course been excluded from the data, the membranes had

been informed by Dr. Landrum B. Shettles (1956) in New York and Drs. Fritz Fuchs and Povl Riis (1956) in Copenhagen, that they have independently found that a diagnosis of foetal sex can be made by an examination of amniotic fluid cells. A report has also appeared from Minneapolis (Makowski, Prem, and Kaiser, 1956), Amsterdam (James, 1956), and Sheffield (Dewhurst, 1956). The diagnosis is therefore well established, and the only possible source of error would seem to be in the rare case of an intersex whose appearance does not correspond to that expected from his or her sex chromosome constitution. Even in cases of twins with different sexes in the same amnion, it should be possible to make a correct diagnosis, since one would expect to find both male and female patches of cells in the amniotic fluid. Regarding the clinical value of a correct prenatal diagnosis of sex, the most obvious application would be in cases of certain sex-linked abnormalities.

TABLE V.—Percentage of Cells With a Chromocentre, in the Amniotic Fluid in 7 Cases in the Sixth and Seventh Month of Pregnancy. Figures in Each Case are Based on a Count of 200 Cells

	Males			Females		
	% Cells with Chromocentre			% Cells with Chromocentre		
	At Nuclear Membrane	Not at Nuclear Membrane	Total	At Nuclear Membrane	Not at Nuclear Membrane	Total
	3.5	4.0	7.5	41.5	11.0	52.5
	4.5	9.5	14.0	34.0	14.0	48.0
	8.0	6.0	14.0	41.0	16.0	57.0
	5.5	7.0	12.5			
Mean	5.4	6.6	12.0	38.8	13.7	52.5
Standard error	0.9	1.1	1.5	2.4	1.4	2.6

presumably broken before the fluid was collected, so that maternal cells were mixed with those of the foetus. Papanicolaou smears, which showed the presence of bacteria, confirmed that there had in fact been maternal contamination in these cases.

The present results show that a diagnosis based on the percentage of cells with a chromocentre at the nuclear membrane gives no less a sex difference than a diagnosis based on the percentage of cells with a chromocentre at any position in the nucleus. The former analysis, which is easier to carry out, is therefore to be recommended as a routine procedure. It should also be noted that the chromocentre of the older epithelial cells found in the amniotic fluid, which may really be a double structure (Sachs and Danon, 1956), is larger when found in a female than when found in a male.

The term "sex chromatin" (Moore, Graham, and Barr 1953) has been used for the chromocentres which are presumably formed by the sex chromosomes. But the term "chromatin" refers to chromosome material in general, so that the sex chromatin of the sex chromosomes is present in all the cells both in males and in females. Since the diagnosis of sex is based not on the presence or absence of sex chromatin but on the different abilities of the sex chromosomes to form chromocentres in the non-dividing nuclei of males and females, the use of the term "chromocentre," or possibly the term "sex chromocentre," seems to be more appropriate than the use of the term "sex chromatin."

Discussion

It can be seen from the present data that an accurate prenatal diagnosis of foetal sex can be made by a study of chromocentres in amniotic fluid cells. In all normal cases there have been a sufficient number of diagnosable cells, and it has been shown that a diagnosis can be made in normal cases from the third month, and possibly even earlier.

Since our original report was published (Serr, Sachs, and Danon, 1955; see also Sachs, Serr, and Danon, 1956) we have

Summary

It has been shown in 40 cases in the ninth month, and in 8 cases in the third, sixth, and seventh months of pregnancy, that it is possible to diagnose the sex of the foetus by a study of chromocentres in amniotic fluid cells. It was also possible to make a diagnosis on an 8-weeks-old aborted foetus.

A clear sex difference has been found both when the diagnosis is based on the percentage of cells with a chromocentre and when it is based on the percentage of cells with a chromocentre at the nuclear membrane. The latter determination is to be recommended as a routine procedure, since it is easier to carry out.

In the 47 cases in the sixth, seventh, and ninth months there was a mean of 4% of cells with a chromocentre at the nuclear membrane in males, and a mean of 35% of such cells in females. The chromocentre was also larger when found in cells of a female than when found in cells of a male.

Three main types of epithelial cells—basal, precornified or cornified, and keratinized—were found in the amniotic fluid. Of these, the basal and precornified cells are the most suitable for the sex diagnosis.

Determinations of the percentage of cells in the amniotic fluid that are suitable for the sex diagnosis gave, for the cases in the sixth, seventh, and ninth months, a mean of 12% suitable cells both for males and for females. In the one case in the third month 61% of the cells were suitable for the diagnosis.

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