DNA CONTENT OF PLACENTAL NUCLEI

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ABSTRACT

The DNA content of individual nuclei in four immature human placentas was determined by microspectrophotometric analysis of Feulgen-stained sections. The absence of mitosis in the syncytiotrophoblast, taken together with the finding of a diploid unimodal distribution, at a time of rapid placental growth, indicated that the syncytiotrophoblast possessed little or no intrinsic reproductive capacity. In contrast, the cytotrophoblast displayed considerable mitotic activity and was found to contain a high proportion of nuclei with DNA values in excess of the diploid amount, corresponding to DNA synthesis in interphase nuclei preparatory to division. From the complementary behavior of the two layers of trophoblast, with respect to evidence of reproductive ability, it is concluded that the rapid accumulation of nuclei in the syncytiotrophoblast, during the early development of the placenta, is accounted for by cell proliferation within the cytotrophoblast followed by alignment and coalescence of some daughter cells in the syncytiotrophoblast.

This investigation was undertaken in an attempt to shed further light on the morphogenic relationship between the two types of trophoblast, cytotrophoblast and syncytiotrophoblast, found in the human placenta. Cytotrophoblast consists of single-nucleated cells, displaying mitotic activity, which invest the villi and compose most of the attaching columns. Syncytiotrophoblast forms a syncytial coating of the cytotrophoblast and thus comprises the unbroken outermost frontier of the conceptus, in direct and intimate contact with the maternal tissues. One of the most striking characteristics of the development of the placenta is the rapid accumulation of large numbers of nuclei in the syncytiotrophoblast in the absence of mitotic figures. The origin of these nuclei has been attributed to either proliferation within the syncytiotrophoblast, presumably by a process of amitosis (1, 2), or derivation from the cytotrophoblast (3). The apparent paradox of an increase in the number of nuclei in the syncytiotrophoblast, with only equivocal evidence of intrinsic nuclear replication, invites a more critical analysis.

The development of a microspectrophotometric method, based on the Feulgen reaction, for determining the amount of deoxyribonucleic acid (DNA) in individual nuclei in tissue sections (4), offers a unique opportunity to approach this problem. The concept of constancy of DNA is now well established and there is ample evidence that nuclear DNA is characteristic of the karyotype (4). Some contradictory evidence is discussed by Chayen (5). Variations occur in a number of well defined physiologic and pathologic conditions, the commonest normal fluctuation resulting from DNA synthesis in interphase nuclei preparatory to nuclear division. When plotted on DNA histograms, the DNA content of such nuclei appear scattered between the circumscribed diploid, or 2n, class and a value of 4n.

The findings of microspectrophotometric analysis of the DNA content of the nuclei in four immature human placentas are presented and from these data the probable mechanism of morphogenesis of the syncytiotrophoblast is deduced.

MATERIAL AND METHODS

Four placentas were selected on the basis of the period of gestation, histologic normality, and preservation of nuclear detail (Table I), from fifteen placentas obtained at the time of therapeutic interruption of pregnancy.

Small fragments of placenta, approximately 2 mm in diameter, were placed within five minutes of excision in three freshly prepared fixatives (10 per cent neutral buffered formalin, Lavdowsky, and Carnoy—1 part glacial acetic acid + 3 parts 100 per cent ethanol). After 4½ hours, the tissues were rinsed and kept overnight in 70 per cent alcohol, and embedded in paraffin (Tissuemat; Fisher, Pittsburgh) (4). The blocks were stored at room temperature for approximately six months.

Sections at 5 μ and 9 μ were prepared and stained by the Feulgen technique (4) (12 minutes hydrolysis in 1 N HCl preheated to 60°C; staining for 2 hours in freshly prepared Schiff reagent in the dark). In addition, a thin Parlodion (purified pyroxylin; Malinckrodt, St. Louis) coating was applied to prevent dislodgment of the sections, which was otherwise liable to occur following hydrolysis. No counterstain was used as it was found that fast green accounted for a small but definite, non-specific, cytoplasmic extinction. The sections were mounted in Permount (Fisher) which was allowed to harden at room temperature. DNA, present only in whole and fragmenting nuclei, stained a bright red–magenta, while the background was colorless. No staining occurred in unhydrolyzed control sections. Although each placenta was fixed and embedded separately, placentas 1 and 2 were stained together, and placentas 3 and 4 were also processed simultaneously, although at another time. There is good agreement between the DNA values of the similarly treated placentas, particularly in the villous stroma.

Representative sections were stained with hematoxylin and eosin, methyl green–pyronin, and some Feulgen-stained sections were counterstained with fast green, for general histologic examination.

The measurement of DNA in individual nuclei was performed on the Feulgen-stained sections, using the technique and apparatus described in detail by Leuchtenberger (4), with the following modifications:

1. The light source was provided by the built-in

TABLE I

Clinical Details of Material Selected for Microspectrophotometric Study

Study number	Pathology specimen number: BLiH S60-	Maternal		Therapeutic Interruption of Pregnancy				
		Age	Parity	Period of gestation	Indication	Route	Gross findings	Fixative
		(*yrs.*)		(*days*)*				
1	2308	44	G6 P4	73	Psychiatric	D. and C.	Placental fragments only; fetal parts not identified.	Carnoy
2	817	46	G3 P2	65	Psychiatric; Hyperemesis; Leiomyomata	Hysterectomy	Diamnionic, monochorionic, twin pregnancy: both male; CR 3.2 cm.	Lavdowsky
3	2504	24	G5 P4	55	Rubella	D. and C.	Placental fragments only; fetal parts not identified.	Carnoy
4	1959	28	G7 P3	49	Psychiatric	D. and C.	Placental fragments only; fetal parts not identified.	Carnoy

* From onset of LMP.

In all four cases the pregnancy was uneventful apart from the presenting condition. There was no history of toxemia, Rh incompatibility, or unexplained abortion complicating earlier pregnancies.

incandescent tungsten lamp (6 volts, 15 watts, Phillips 13347W), operated at 5 volts a-c;

2. A Corning narrow band pass filter (Color Specification 4–120, consisting of 1–60, 3–69, and 4–96, 2-inch-square polished glasses cemented together) was placed over the window, through which the light path emerged, in the microscope base. Filter characteristics: peak transmission 44 per cent; $\lambda_{max.T}$ = 546 mμ; 50 per cent $T_{max.}$ = T_{537} = T_{565}; 1 per cent $T_{max.}$ = T_{505} = T_{572};

3. A two-diaphragm, brightfield, substage condenser was used. Both the lower, or field, diaphragm and the aperture diaphragm were stopped down completely to diminish stray light and glare. The effective numerical aperture was somewhat less than 0.2. Köhler illumination was used, with a field of 100 μ diameter.

The amount of DNA *per* nucleus, expressed in arbitrary units, was determined by the method of Swift (6). Nuclear volume was calculated on the basis of "average axis" (7).

All measurements were performed in a dark room, both to avoid errors due to light leaks in the apparatus and to facilitate observation of the dim, magnified, telescope image. Individual villi were scanned systematically and suitable nuclei were measured, twenty at a time, in the syncytiotrophoblast, cytotrophoblast, and villous stroma in sequence until a total of 200 nuclei from each tissue had been measured. In the villous trophoblast, only nuclei in the clearly defined layers were measured (Figs. 1 *A*; 1 *B*); the nuclei in syncytial buds were ignored.

For valid comparison, all measurements for each placenta were made in one microscopic section by a single observer. A compromise section thickness of 9 μ provided the optimum between cutting of the larger cytotrophoblast nuclei and overlapping of the smaller and more crowded syncytiotrophoblast nuclei. Careful appraisal of consecutive nuclei allowed the inclusion of only whole, discrete, spheroid nuclei of suitable shape and dye homogeneity. All cut or overlapped nuclei were rejected, as were irregularly shaped nuclei, nuclei with more than a slight degree of inhomogeneity, and spheroid nuclei with a ratio of short to long axes less than 0.6, the latter on account of the error introduced by using formulae based on spherical shape to calculate the fraction of total volume occupied by the central cylinder in the case of such ellipsoids (8).

RESULTS

Microspectrophotometric Measurements

DNA values of nuclei in placenta 1, plotted separately along the same abscissa to facilitate comparison, are shown in the form of a histogram in Fig. 2. The individual histograms show two distinct nuclear classes, the predominant diploid group and a variable number of nuclei scattered in the higher ranges, the interclass group. Characterization of each cell population is achieved by the calculation of the mean and standard deviation of the diploid group, using formulae based on correspondence to a normal curve.

Comparison of the general form of the complete histograms shows a relatively uniform distribution in the diploid range in each instance, with similar values of standard deviation, 0.29, 0.28, and 0.21 units (arbitrary) in the syncytiotrophoblast, cytotrophoblast, and villous stroma respectively. In contrast, there is a sharp disparity in the number of nuclei in the interclass groups, varying from only 2 per cent in the syncytiotrophoblast to 17 per cent in the cytotrophoblast with an intermediate number of 5 per cent in the villous stroma. Similar observations were made in the other three placentas (Table II).

The diploid mean value of the syncytiotrophoblast is 9.5 per cent less than the equivalent stromal value ($P < 0.001$). Significant differences varying from 9.1 to 14.1 per cent, average 10.7 per cent ($P < 0.001$ in each case), are consistently present in each placenta (Table II).

A considerable proportion, 50 per cent or more, of nuclei with DNA content between 4 and 5 units, corresponding to a 4n and possibly tetraploid group, was present in a small number of nuclei measured in the cytotrophoblast columns. Nuclei possessing duplicate Barr chromatin bodies were commonly observed in these regions.

Nuclear Size

Fig. 3 presents a composite histogram of volume, based on "average axis" and the formula for a sphere, of nuclei in the diploid groups in placenta 1. In general, the interclass DNA groups comprise the largest nuclei and their omission from the volume histograms leads to somewhat more normal distributions. Within the diploid range there is little relation between DNA content and nuclear volume, as is shown by the low values of the coefficients of correlation (r). In the case of the syncytiotrophoblast, even these low values of r tend to be exaggerated by the skew distribution of nuclear volume. Although part of this asymmetry is due to the effect of ignoring nuclei with a diameter less than 4 μ, corresponding to the limiting factor imposed by the plug size, it can be seen that a nor-

mal distribution is impossible owing to proximity to zero.

The mean values of nuclear volume of nuclei in the diploid DNA groups show considerable conformity between the same tissues in the four placentas (Table III). The average values of the villous stroma and cytotrophoblast are 26 per cent and 61 per cent, respectively, higher than that of the syncytiotrophoblast.

DISCUSSION

Two salient features emerge as a result of this investigation: the similarity of the general distribution of DNA diploid class values and the marked differences of interclass size. From these data deductions can be made as to whether nuclear replication in the syncytiotrophoblast occurs by a process of random fragmentation or by orderly mitotic division.

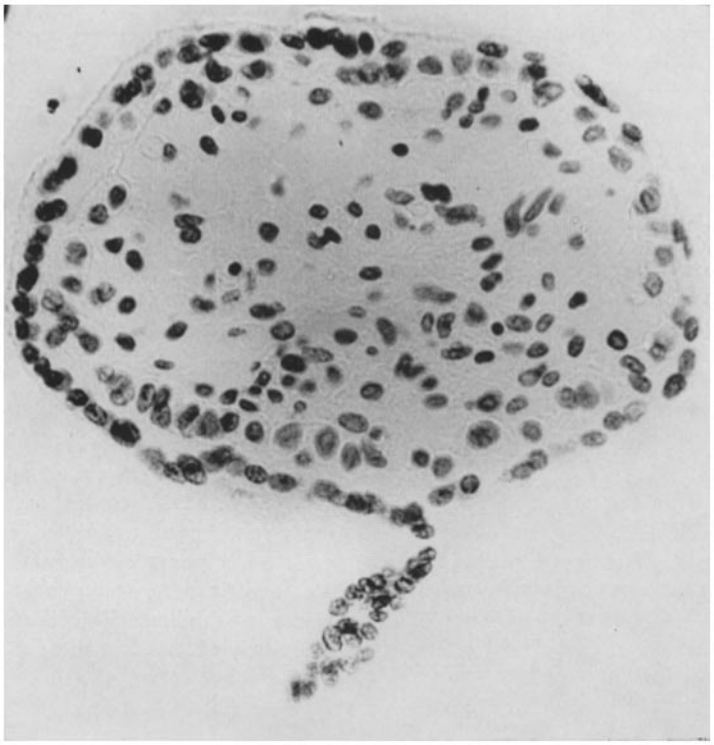

FIGURE 1 *A*

Cross-section of a villus showing the two layers of trophoblast, with a syncytial bud, and the cellular stroma. (Placenta 1, 9 μ, Feulgen and fast green, green filter, × 640.)

Mitotic Index

The mitotic index, expressed as the *per mille* proportion of the population in any mitotic stage, was determined in the same sections that were used for DNA measurement. The mitotic index is roughly proportional to the interclass group size (Table IV). The possession of the Barr nuclear chromatin body is recorded in Table IV, together with the presumptive fetal sex.

Most syncytiotrophoblast nuclei fall into one well defined class corresponding to the diploid amount of DNA, with the exception of the 1½ to 4 per cent which exceed the diploid quantity. This pattern is in sharp contrast with the measurements of both the cytotrophoblast and stroma in which an average of 18 and 7 per cent, respectively, of the nuclei fall outside the diploid class. In view of the degree of agreement provided by the concomitant changes of mitotic index, it seems

likely that the nuclei with DNA content in excess of the diploid value are undergoing DNA synthesis in preparation for cell division (6, 9).

In so far as it is unlikely that any form of nuclear replication could occur in the absence of DNA synthesis, the presence of a single unimodal distribution of DNA values in the syncytiotrophoblast appears to preclude equally the possibilities of mitosis and amitosis. Mitosis is excluded by the rather remote, alternative of postdivision synthesis, which in turn can be disregarded owing to the narrow distribution of DNA values: a wide scatter ranging from at least 1n to 2n would be expected on the basis of nuclear division followed by DNA synthesis.

The interclass values (1½ to 4 per cent) in the syncytiotrophoblast may be considered as due to either biological variation or measuring error,

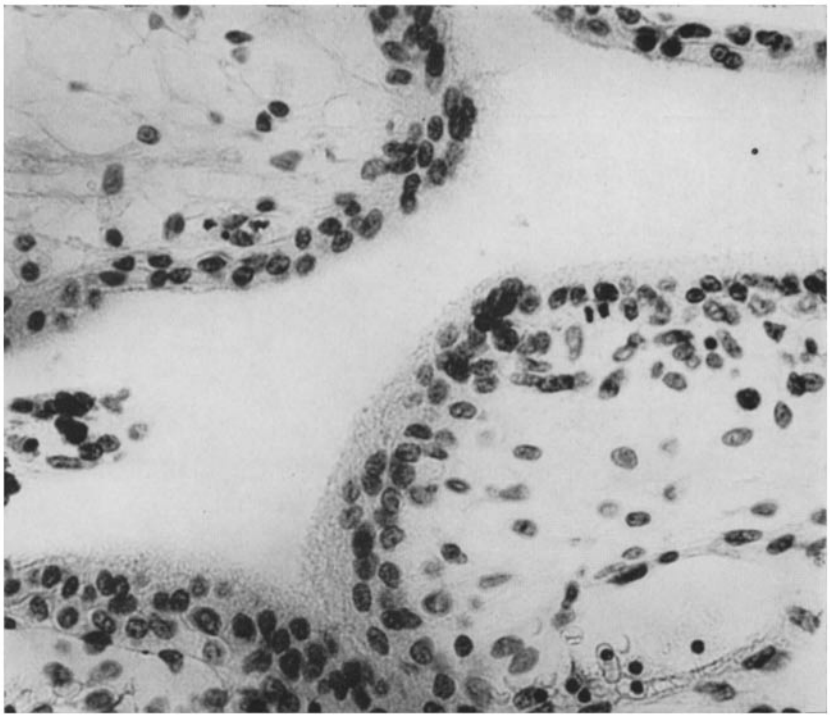

FIGURE 1 *B*

Trophoblast of neighboring villi border the intervillous space, now empty but which normally contains maternal blood. A syncytial bud is present. A mitotic figure can be seen in the cytotrophoblast, and nucleated fetal red blood cells are evident in a fetal capillary deep in the stroma. (Placenta 1, 9 μ, Feulgen and fast green, green filter, × 640.)

paucity of interclass values, indicative of virtual absence of predivision DNA synthesis. This conclusion is reinforced by the statement of all investigators regarding the absence of mitotic figures in the syncytiotrophoblast (1, 3, 10). Similarly, amitosis, if it is to imply a truly reproductive process, as opposed to progressive fragmentation with unchanged chromosomal mass, must include a period of DNA synthesis. Predivision synthesis has already been ruled out, leaving the only, and the latter possibility being caused by the occasional inclusion of an unrecognized overlapped nucleus. If they are assumed to be biologically significant, then they may be regarded as evidence for a low level of DNA synthesis in the syncytiotrophoblast. In this case, it would be pure speculation to comment on whether such synthesis is associated with subsequent nuclear division. Alternatively, this small proportion of nuclei may represent the product of abnormal mitosis occurring in the cyto-

trophoblast before nuclear alignment in the syncytiotrophoblast.

The complementary behavior of the two layers of trophoblast, with respect to reproductive activity, strongly suggests that the syncytiotrophoblast is continuously arising as a result of coalescence of cytotrophoblast daughter cells.

tion. Electron microscopic studies have illustrated the persistence of villous cytotrophoblast in normal term placentas (11), while histologic examination of such material following careful preparation reveals the presence of mitotic figures (12).

Before a definitive statement can be made regarding the morphogenesis of syncytiotrophoblast,

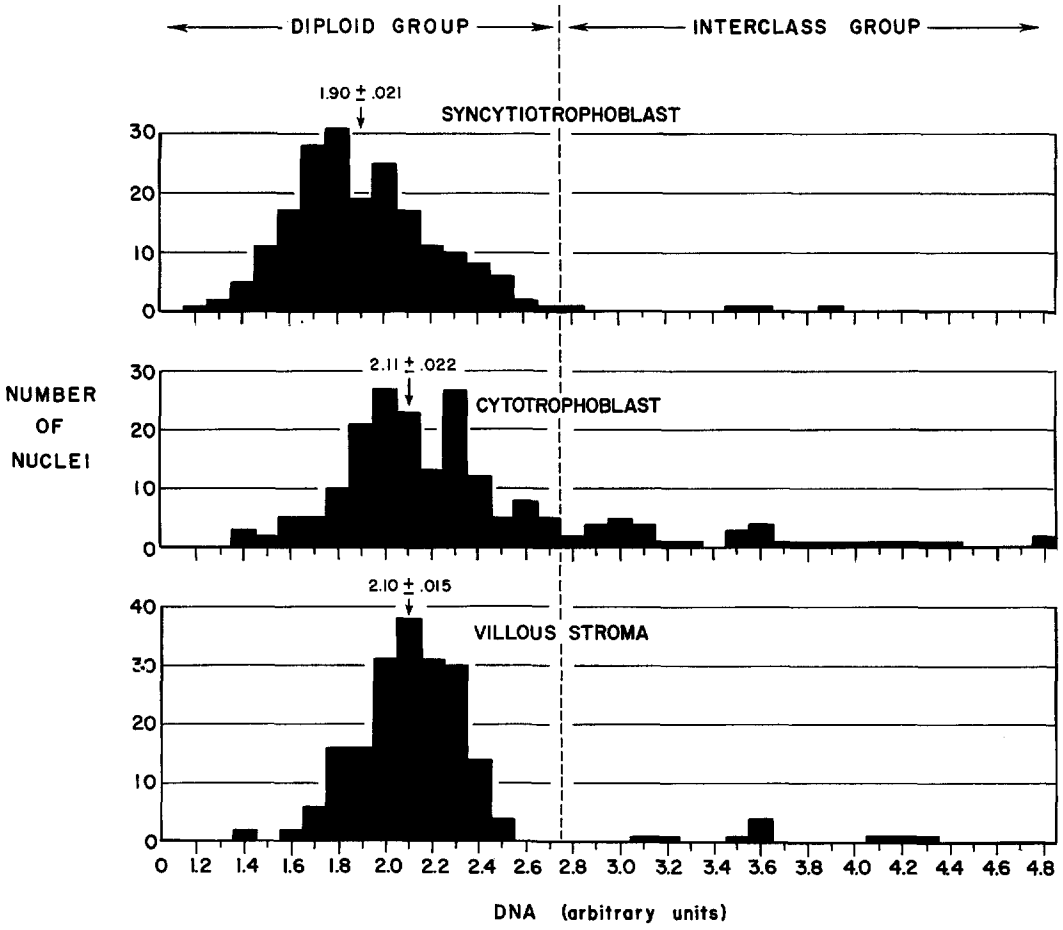

FIGURE 2

Composite histogram of nuclear DNA content (Placenta 1).

During the period of development studied, the intense mitotic activity of the cytotrophoblast would appear to be adequate to account fully for the rapid growth of both layers of trophoblast. The initial evolution of the syncytiotrophoblast is accomplished in this manner (1, 3, 10), and it is possible that the same process is operative throughout pregnancy, despite the considerable attrition and subsequent apparent inactivity of the cytotrophoblast commencing at 12 to 14 weeks gestation. Electron microscopic studies have illustrated the persistence of villous cytotrophoblast in normal term placentas (11), while histologic examination of such material following careful preparation reveals the presence of mitotic figures (12).

Before a definitive statement can be made regarding the morphogenesis of syncytiotrophoblast, further studies will be necessary using such techniques as time-lapse cinemicrography and suitable nuclear labeling (13, 14).

The slight gradient of diploid group mean DNA values in each placenta, with the syncytiotrophoblast consistently occupying the lowest position (Table II), could conceivably be due to actual loss of DNA from syncytiotrophoblast nuclei. In view of the difficulty in assessing the contributions of a number of potential sources of error (15), par-

TABLE II

Comparison of DNA Measurements

Placenta	Syncytiotrophoblast		Cytotrophoblast		Villous stroma	
	Mean diploid value ± s.e.*	Interclass group size	Mean diploid value ± s.e.*	Interclass group size	Mean diploid value ± s.e.*	Interclass group size
		%		%		%
1	1.90 ± 0.021	2	2.11 ± 0.022	17	2.10 ± 0.015	5
ratio	90.5		100		100	
2	1.83 ± 0.021	4	1.91 ± 0.023	8	2.13 ± 0.020	9
ratio	85.9		89.7		100	
3	2.22 ± 0.021	1.5	2.36 ± 0.023	19.5	2.47 ± 0.016	6
ratio	89.9		95.5		100	
4	2.21 ± 0.016	2	2.43 ± 0.019	20	2.43 ± 0.013	8
ratio	90.9		100		100	

* Arbitrary units.

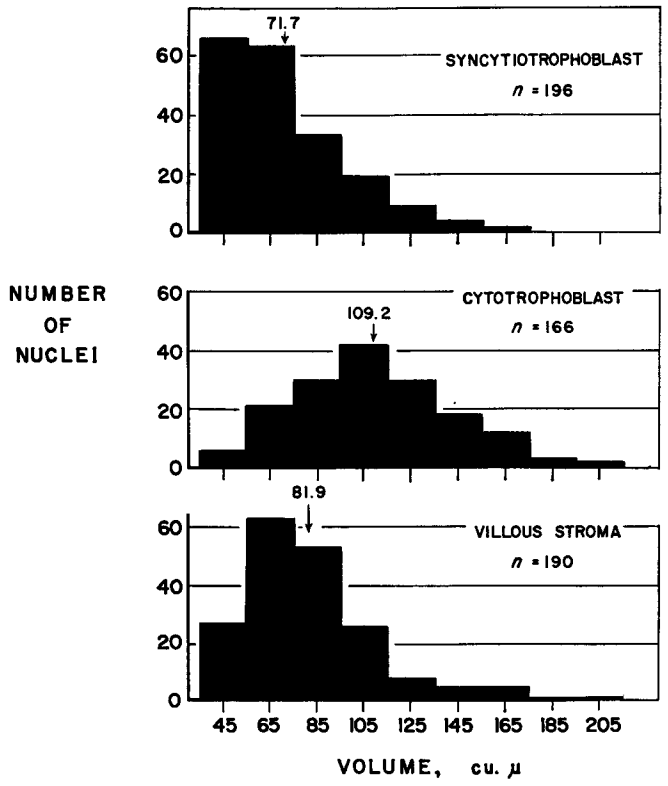

FIGURE 3

Composite histogram of nuclear volume of nuclei in diploid DNA groups (Placenta 1).

TABLE III

Comparison of Nuclear Size

(Nuclei in diploid DNA groups)

Placenta	Syncytiotrophoblast		Cytotrophoblast		Villous stroma	
	Mean volume ± s.e.	r ± s.e.*	Mean volume ± s.e.	r ± s.e.*	Mean volume ± s.e.	r ± s.e.*
	(*cu. μ*)		(*cu. μ*)		(*cu. μ*)	
1	72 ± 2.0	0.300 ± 0.065	109 ± 2.6	0.238 ± 0.074	82 ± 2.1	0.165 ± 0.071
ratio	100		151		114	
2	81 ± 2.2	0.328 ± 0.064	133 ± 3.3	0.247 ± 0.069	108 ± 3.1	0.132 ± 0.073
ratio	100		164		133	
3	77 ± 1.7	0.253 ± 0.067	125 ± 3.1	0.187 ± 0.076	102 ± 2.3	0.188 ± 0.070
ratio	100		162		132	
4	79 ± 1.7	0.454 ± 0.057	128 ± 2.5	0.180 ± 0.076	96 ± 2.1	0.258 ± 0.069
ratio	100		162		122	
Average ratio	77		124		97	
	100		161		126	

* r = correlation coefficient between volume and DNA content of nuclei in the diploid group.

standard error of $r = \frac{1 - r^2}{\sqrt{N - 2}}$.

TABLE IV

Mitotic Index, Interclass Group Size, and Barr Nuclear Chromatin

Placenta		Syncytiotrophoblast	Cytotrophoblast	Villous stroma	Barr nuclear chromatin; presumptive fetal sex
1	Mitotic index*	0	9	4.5	Absent—♂
	Interclass group size, %	2	17	5	
2	Mitotic index*	0	6	5	Absent—♂
	Interclass group size, %	4	8	9	
3	Mitotic index*	0	11.5	7	Absent—♂
	Interclass group size, %	1.5	19.5	6	
4	Mitotic index*	0	11.5	5	Present—♀
	Interclass group size, %	2	20	8	

* *per mille.*

ticularly those associated with light scatter and related phenomena (16), it is impossible to attribute any real significance to this finding. The use of more refined techniques, such as integration scanning and two-wavelength methods, will be necessary to verify the existence of these small differences.

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