PARENTAL ORIGIN OF TRIPLOIDY AND TRISOMY IN HUMAN MISCARRIAGES

BY

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ABSTRACT

Caspersson (1970) discovered that each chromosome pair has a characteristic banding pattern when chromosomes are stained with the fluorescent dye, quinacrine. With this dye there are certain chromosome regions which are polymorphic. These regions can be used as markers in the study of the inheritance of chromosome anomalies.

The purpose of this study was to determine, using chromosome markers, the parental origin of the extra chromosomes found in triploid and D, G trisomic spontaneous abortuses. Polymorphisms in the chromosomes of parents of 15 triploid and 12 trisomic abortuses were compared with those of their abortus to determine which parent donated the extra chromosome(s). The distribution of the markers was used to determine whether the error occurred during meiosis I or meiosis II or, in the case of triploids, to distinguish between a meiotic error and dispermy.

Of the 15 triploids examined, 7 were informative as to the origin of the extra set of chromosomes. In 3 of these there was failure to extrude to second polar body during oogenesis. In 3 other cases it was impossible to distinguish between an error during meiosis of spermatogenesis and dispermy. The other informative case

definitely arose by dispermy. Mechanisms for the origin of triploidy were discussed, in particular, aging of gametes.

Only 1 of the 12 trisomies examined was informative. This was a trisomy 22 in which the extra 22 was from the mother but it was not possible to distinguish between non-disjunction during meiosis I and meiosis II. Possible mechanisms for the production of trisomies were discussed including maternal irradiation, autoimmune processes, possible endocrine factors and drugs.

A number of technical factors which influence the appearance of polymorphic regions were also discussed.

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INTRODUCTION

I. Historical Perspective of Technical Developments

In 1956, Tjio and Levan demonstrated with the use of cultures of fetal lung tissue that the human diploid chromosome number is 46. This number was confirmed in 1956 by Ford and Hamerton who counted 23 bivalents in meiotic material from testicular biopsies. The determination of the human chromosome complement resulted from the application of techniques from plant genetics to human fibroblasts. One of these techniques was the use of colchicine to arrest cells in metaphase. Hsu (1952) discovered accidentally that hypotonic treatment of cells before fixation led to the swelling of the cells and created much better chromosome spreads. The common hypotonic treatment used was sodium citrate. Tjio and Levan (1956) employed both of these techniques in their examination of the chromosomes of lung fibroblasts. Several weeks are needed to obtain sufficient growth of fibroblasts. This is the major disadvantage of such culture.

Simultaneously with the above workers, other researchers were attempting to culture cells from peripheral blood. Several unrelated discoveries led to the eventual success of this technique. Nowell (1960a)

showed that peripheral blood cultures exhibit a burst of mitotic activity after 48-72 hours in culture. In preparing cultures, Nowell used phytohaemagglutinin (PHA) to separate leukocytes from red cells. When he investigated systematically which factor of his culture technique was the initiator of this mitotic activity he found that the PHA was responsible (Nowell, 1960b). This was a very important discovery because the number of cells undergoing spontaneous mitosis in peripheral blood cultures is small. It also enabled more rapid preparation of chromosomes than with fibroblast cultures.

In 1960, Moorhead combined Nowell's culture techniques with air-drying of slide preparations (Rothfels and Siminovitch, 1958), in place of the squash methods used previously. This procedure also improved chromosome spreads. Hungerford (1965) used 0.075 M KCl as the hypotonic pretreatment for cells.

This combination of techniques gave rise to a very successful culture method for the small lymphocytes present in peripheral blood and chromosome preparation. Using this method the number of chromosomes in a preparation could be easily counted. However, it was still not possible to distinguish all of the chromosome pairs from each other.

II. Banding Human Chromosomes

The field of human cytogenetics progressed no further until Caspersson discovered a technique for chromosome banding. Prior to this, few chromosomes could be identified unequivocably. Most could be assigned only to one of the 7 groups (labelled A to G) which were based on similarities in chromosome length and position of the centromere.

Caspersson et al. (1969a) treated preparations of Vicia faba root tips with the fluorescent dye quinacrine mustard to study the process of differentiation. They hoped to identify physico-chemical differences between regions of metaphase chromosomes. With quinacrine mustard they obtained fluorescent bands on each chromosome which were identical on sister chromatids. The bands were characteristic for each chromosome and were reproducible. Caspersson et al. (1970) stained leukocyte preparations of human chromosomes with quinacrine mustard and found that again a consistent banding pattern was produced for each chromosome. A photometric technique was used to identify the 24 fluorescence patterns of metaphase chromosomes (Caspersson et al., 1971). A number was assigned to each chromosome pair. On the basis of chromosome length, centromere-index, late-labelling pattern (especially for chromosomes 13, 14 and 15) and

banding properties all chromosome pairs were uniquely identified. The chromosome which is trisomic in Down syndrome was called 21 because of the number of years that the extra chromosome was considered to be 21 despite the fact that 22 is slightly longer.

At the Paris Conference (1971) the quinacrine banding characteristics for each chromosome pair were outlined in detail as an international standard. A band was defined as "a part of a chromosome which is clearly distinguishable from its adjacent segments by appearing darker or lighter with one of the staining methods".

Miller et al. (1971) employed Caspersson's findings in the characterization of structural chromosome anomalies. They used banding on the chromosomes of patients with translocations and demonstrated that the translocation chromosomes retained the quinacrine fluorescence patterns of the chromosomes from which they were derived. Lin et al. (1971) and Evans et al. (1971) described methods for the use of quinacrine dihydrochloride, instead of quinacrine mustard which was difficult to obtain. Quinacrine dihydrochloride enabled potential routine examination of chromosomes in cytogenetics service laboratories.

A number of other banding techniques are now being used in addition to quinacrine. These include: Giemsa (G)

banding (Seabright, 1971; Sumner et al., 1971); reverse (R) banding (Dutrillaux and Lejeune, 1971); centromeric heterochromatin (C) banding (Arrighi and Hsu, 1971); a modification of R banding with acridine orange (Bobrow and Madan, 1973); telomeric (T) banding (Dutrillaux, 1973); and daunomycin (D) banding (Lin and Van de Sande, 1975).

The major question yet to be answered is how the bands are produced. Since quinacrine mustard binds to the N7 of guanine, Caspersson et al. (1969b) suggested that chromosome bands represent guanine-rich regions of deoxyribonucleic acid (DNA). However, quinacrine dihydrochloride which produces comparable bands to quinacrine mustard is a non-alkylating compound, that is, it does not bind to the N7 of guanine. Moreover, Sumner et al. (1973) blocked the N7 of guanine using mustard gas and still produced the characteristic bands. This evidence suggests that chromosome bands do not represent guanine-rich regions of the DNA. Freeman (1971) described a method by which he produced antinucleoside antibodies specific for each of the 4 bases in DNA. These fluorescein-tagged antibodies reacted only with single-stranded DNA. Dev (1972) used these antinucleoside antibodies to show that binding of quinacrine dihydrochloride is not directly related to the DNA base sequence. Antibodies against adenosine and cytidine both produced banding patterns similar to those of quinacrine.

Weisblum and de Haseth (1972) observed quenching of fluorescence with increasing numbers of guanine-cytosine (GC) pairs using several microbial DNA's. Thus, the importance of the DNA base sequence to quinacrine binding is not clear. There may be several interacting factors so that it is not possible to ascertain their relative importance.

Caspersson et al. (1969b) suggested that the brightly fluorescent bands produced with quinacrine represent heterochromatin. Heterochromatin is tightly coiled in mitotic prophase in contrast to euchromatin which is loosely coiled. Because of this tight coiling heterochromatin undergoes DNA replication late in S phase. Two types of heterochromatin have been described: constitutive and facultative. Ganner and Evans (1971) suggested that the fluorescent bands more likely represented constitutive rather than facultative heterochromatin. Constitutive heterochromatin contains DNA with highly repetitive sequences. It is found in the same location in both members of a homologous pair of chromosomes. An example is that chromatin found in the centromere regions of most chromosomes. Facultative heterochromatin is not found in both members of a homologous pair and does not contain repetitive sequences. An example of facultative heterochromatin is one of the two X chromosomes in human females. Early in embryonic development one X becomes heterochromatin-

ized (inactivated). Since both X's band identically with quinacrine this implies a more condensed structure for the inactive X, not a change in organization such as the repeated sequences of constitutive heterochromatin. Ganner and Evans (1971) performed autoradiography and then stained the same cells with quinacrine. They found that the regions of bright fluorescence showed a high rate of incorporation of ^{3}H thymidine late in S phase. However, the secondary constrictions of chromosomes 1, 9 and 16 were late-replicating yet were not brightly fluorescent.

The quinacrine banding pattern is not abolished by the removal of ribonucleic acid (RNA) with RNAase (Comings, 1971). When the DNA is removed with DNAase there is no staining with quinacrine (Sumner et al., 1973). Sumner et al. (1973) also showed that methanol-acetic acid fixation, an integral part of chromosome preparation, removes a large amount of protein, especially histones. Therefore, histone proteins are not responsible for banding. The presence of the histones may suppress the binding of the fluorescent dye.

Comings (1971) suggested that staining patterns may reflect binding to different regions of the genome of varying amounts of non-histone proteins. Non-histone proteins are involved in the regulation of genetic activity and are tissue and species specific. Caspersson et al. (1972)

found identical chromosome banding patterns in cells of amnion, bone marrow, skin and testis. Therefore, non-histone proteins are not solely involved in the production of bands.

Sumner et al. (1973) proposed a theory which they felt accounted for the conditions necessary for the production of chromosome bands by quinacrine. They proposed that the dye molecule combined ionically with phosphate groups and that most dye will be bound when phosphates are the correct distance apart to bind both amino groups of the dye molecule.

The evidence discussed suggests that there are a number of factors involved in the production of chromosome bands by quinacrine. The DNA base sequence is not directly involved. The areas of bright fluorescence are constitutive heterochromatin with the exception of the secondary constrictions of chromosomes 1, 9 and 16 which are heterochromatic but not brightly fluorescent. Non-histone protein -DNA interactions are probably involved and histone proteins are not necessary for the production of bands. The exact mechanism of the production of bands by quinacrine is yet to be elucidated.

III. Human Chromosome Polymorphisms

With the advent of chromosome banding techniques came the observation that, especially with quinacrine fluorescence, there are certain regions of the human chromosome complement that are polymorphic, that is, they may vary from one individual to another. These polymorphic features are normal chromosomal variants which are inherited in a Mendelian manner (Buckton, 1976). Quinacrine fluorescence polymorphisms were first described by Caspersson et al. (1971) and confirmed by Evans et al. (1971). Two groups of chromosomes which exhibit polymorphisms are the large and small acrocentrics. Variable features are the short arms, stalks and satellites. The stalks, which may vary in length, have been shown by Goodpasture et al. (1976) to be the nucleolar organizing regions (NOR) of the human chromosomes, that is, the stalks contain the genes for ribosomal RNA. The short arms and the satellites of the acrocentric chromosomes vary both in their intensity of fluorescence and in their size. However, it is difficult to separate these two variables since the satellite region may appear larger merely because it is intensely fluorescent. Another chromosome containing a polymorphic region is chromosome 3. It may or may not have a bright band near the centromere on the long arm. In certain individuals the band is present on the short

arm. Chromosome 4 may or may not possess a bright centromeric band. The length of the secondary constrictions on chromosomes 1, 9 and 16 may also vary. Chromosome 17 may occasionally possess satellites. Thus, chromosomes 1, 3, 4, 13, 14, 15, 16, 17, 21 and 22 may be used as markers.

Mikelsaar et al. (1974) obtained the frequencies of these polymorphic regions among Estonians. The presence of bright bands on chromosomes 3, 4 and 13 had a frequency of 64.9, 27.6 and 84.4% respectively. They found a sex difference with respect to homo- and heterozygotes for chromosome 3 only, with women being more often heterozygous and men homozygous. Similar studies have been conducted on newborn populations (McKenzie and Lubs, 1975; Muller et al., 1975; Lin et al., 1976). Although the exact frequencies obtained by these groups are not identical to those of Mikelsaar et al. (1974), their relative frequencies are similar. It is not possible to make meaningful comparisons between the groups because the classification and method of scoring of variants differed for each group. Another compounding factor is the difficulty in assessing quantitatively fluorescent intensity.

In 1975 (Paris Conference, Supplement) it was suggested to standardize human cytogenetic research by scoring chromosome regions with variable fluorescence

according to 5 intensity levels. These were: brilliant (5), intense (4), medium (3), pale (2) and negative (1). There are a number of technical difficulties associated with this attempt at a very precise method of classification. It is not easy to distinguish between the presence of satellites of low intensity and the absence of satellites. In addition, where satellites are definitely present the level of intensity may be difficult to assess. The quality of fluorescence may vary from photograph to photograph depending on the quality of the negative and the exposure time. To overcome these technical difficulties, scoring from direct observation is possible. However, for documentation, photographs are necessary. For each picture it may be possible to rate markers on a scale of 1-5 but the rating is a somewhat subjective decision. As long as these limitations are realized, human chromosome polymorphisms can be of value in studying the inheritance of extra chromosomes found in trisomies and triploids.

IV. Triploidy and its Origin

Penrose and Delhanty (1961) were the first to describe a spontaneously aborted fetus with an abnormal karyotype. This fetus was shown to possess 69 chromosomes. Triploidy has since been found to occur in approximately 20% of spontaneous abortions with an abnormal karyotype (Geneva Conference, 1966; Carr, 1967; Arakaki and Waxman,

1970). This represents approximately 1% of all conceptions in man. Makino et al. (1964) first noticed that a triploid spontaneous abortus possessed hydatidiform degeneration of the chorionic villi. Carr (1969) reported that if hydatidiform degeneration is present there is a 70% chance that the conceptus is triploid. The presence of triploidy is associated with hydatidiform degeneration or true mole in 85% of cases. Carr (1970) found that the incidence of triploidy was increased 4.5 times over controls when women conceived within 6 months of discontinuing oral contraceptives but Boué et al. (1975) did not find this increase in triploid conceptuses.

Although most triploid conceptuses are aborted by the end of the first trimester (Carr, 1971), a number of cases of triploids surviving past this time have been reported, some of whom were live-born (Uchida and Lin, 1972; Walker et al., 1973; de Grouchy et al., 1974; others reviewed by Saadi et al., 1976). By combining the information concerning malformations found in these triploids it has been possible to describe the triploid syndrome (Wertelecki et al., 1976). The most common features found were low birthweight, severe eye defects, syndactyly, dysplastic cranial bones, genital abnormalities in males, omphalocoele, facial clefts and a number of less frequent anomalies. In addition to fetal abnormalities,

triploid gestations may cause maternal complications such as pre-eclampsia and hydramnios (Gosden et al., 1976).

At the cellular level, Mittwoch and Delhanty (1972) showed that triploid cells grown in culture had prolonged cell division time compared to cells with a normal karyotype. This disturbance of growth may partially explain the lethality of this condition.

Junien et al. (1976) measured the activity of several autosomal and X-linked enzymes in cultures of human triploid cells. The cells were 69,XXY and there was no Barr body which indicated both X chromosomes were functioning. Their biochemical evidence also supported the existence of two functional X chromosomes. XXY triploids would be expected to have either one X chromatin body per cell or none. In other words, cells may have either 1 or 2 active X chromosomes. Weaver and Gartler (1975) compared the clinical features of XXY triploids surviving 31 weeks gestation whose cells contained 1 or 2 active X chromosomes. They found no significant differences between triploids which were sex chromatin negative and those which were sex chromatin positive, indicating that the presence of 2 functional X chromosomes is not responsible for the observed abnormalities.

There are several ways in which a triploid conceptus could arise: fertilization of a diploid ovum by

a haploid sperm, fertilization of a haploid ovum by a diploid sperm or by two sperms. The former case is called digyny and the latter two, diandry. A diploid sperm or ovum can result from an error during either the first or second meiotic division. It is possible to distinguish between certain of these mechanisms with the aid of chromosome polymorphisms. Digyny with failure of chromosome separation during the first meiotic division would result in chromosome markers which correspond to two markers found in the mother for a given chromosome. However, if the error occurred in the second meiotic division the abortus would possess two chromosomes with the same marker. This would be true for any chromosome containing a polymorphism. The underlying assumption is that crossing over does not occur between the marker and the centromere. Robinson (1973) suggested that such crossing over is unlikely because of the proximity of the centromere to the marker.

In cases of diandry it is sometimes possible to discriminate between fertilization by a diploid sperm and dispermy. This is difficult but the sex chromosomes may be of some assistance. For example, if the sex chromosomes were XXY and the extra set of chromosomes shown to be of paternal origin by marker studies, then a diploid sperm could only be XY. This would result from an error during

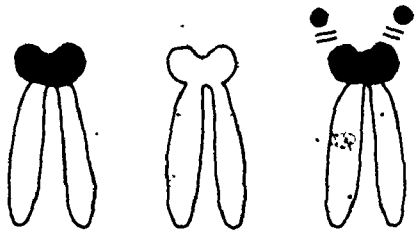
the first meiotic division since it is this division which separates homologues. If this were the case, then the markers for whatever chromosome chosen should be identical to those in the father. This would be useful only if the father is heterozygous for a marker, as illustrated in Fig. 1. This XXY triploid could also have arisen by fertilization of a haploid ovum by two sperms, one bearing an X chromosome and one a Y chromosome. If the triploid had one of each marker found in the father, then it is not possible to determine with certainty whether dispermy or fertilization with a diploid sperm occurred. However, by examining several chromosome pairs it is possible to tell which of the two mechanisms is more likely. If the father was heterozygous for several markers then by dispermy the triploid would be expected to have a random assortment of these markers. Using this reasoning it should be possible in certain instances to determine whether the triploid arose by dispermy or by failure of one of the meiotic divisions during either spermatogenesis or oogenesis.

There have been several reports in the literature of cases in which attempts were made to determine the origin of triploids using chromosome banding techniques. Uchida and Lin (1972) presented good evidence that an XXY triploid arose by fertilization of an ovum with either

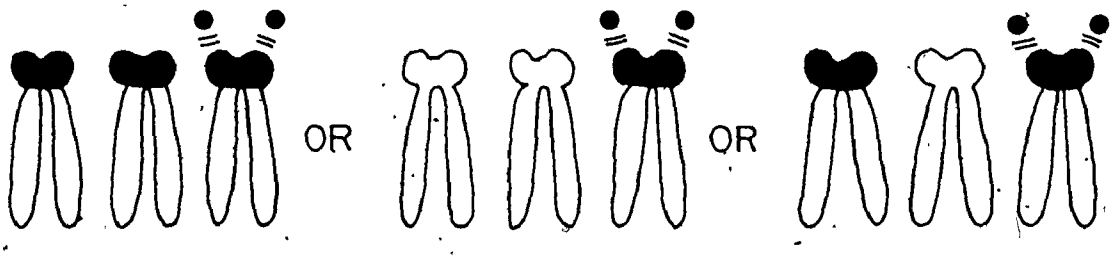
Figure 1: Illustration of the difference in marker chromosomes between diandry caused by a diploid sperm and dispermy, when the sex chromosomes of the triploid are XXY.

HYPOTHETICAL MARKER CHROMOSOME

TRIPLOID RESULTING FROM FAILURE OF MEIOSIS I IN THE FATHER



TRIPLOID CAUSED BY DISPERMY



a diploid sperm or by two sperms. McConnell and Carr (1975) found that in two triploids the extra set of chromosomes were derived from the father. Lauritsen (1976) examined 14 triploid abortuses and the chromosomes of their parents. He found that in 2 cases non-reduction occurred at the first meiotic division in the mother. In a third abortus the extra chromosomes were of paternal origin. In the remaining 11 cases it was not possible to distinguish between maternal and paternal events. Kajii and Niikawa (1977) found that 10 out of 16 triploid abortuses were informative when they used sequential Q and R banding. Of these 10, 5 resulted from dispermy, 2 derived from an error during either the paternal second division or the first mitotic division, 1 was caused by failure of the first meiotic division in the mother and 2 were paternal but no more information could be derived concerning the mechanism. Dewald et al. (1975) reported a 46,XX/69,XXY mosaic in which cytogenetic results showed that double fertilization had occurred. An earlier mosaic reported by Ferrier et al. (1964) seemed to arise by failure of the second meiotic division in the mother. They arrived at this decision because of the presence of two acrocentric chromosomes with large satellites in the triploid mosaic. Only the mother was found to have such a satellited chromosome.

The purpose of this study was to examine the triploids from a selected series of spontaneous abortions in order to determine which parent donated the extra set of chromosomes and secondly, if possible to determine whether the error was more likely to occur during meiosis I or meiosis II.

V. Trisomies and Their Origin

The autosomal trisomies represent approximately 40-50% of first trimester spontaneous abortions with an abnormal karyotype. Of these about 40% involve the D or G group chromosomes (Arakaki and Waxman, 1970; Carr, 1972; Boué et al., 1975). It is well known that advanced maternal age is associated with an increased incidence of the D and G trisomies whether live-born or aborted spontaneously (Boué et al., 1975). These are represented at birth by trisomy 22, trisomy 21 and trisomy 13, all of which possess well-defined clinical features. The maternal age influence on trisomies is important for the acrocentric chromosomes and also for the X chromosome (Ferguson-Smith et al., 1964). Evans (1967) suggested that, because the acrocentric chromosomes are involved in the organization of the nucleolus, any factor, such as aging of oocytes, which may interfere with the dispersion of the nucleolus could account for this increase in non-disjunction.

Cooke (1972) observed a decrease in the number of nucleolar associations with age and suggested that this may be associated with fewer nucleoli in which case those that are present may persist longer in order to produce the same amount of rRNA.

Theoretically non-disjunction can occur in the first or second division during either spermatogenesis or oogenesis. Licznerski and Lindsten (1972) presented the first evidence using chromosome banding that the extra chromosome 21 in a Down syndrome child was inherited from the mother and probably the non-disjunction occurred during the first meiotic division. In this study, only 1 of 6 families was informative. Since this original report there have been a number of studies reporting the origin of the extra chromosomes in autosomal trisomies. Langenbeck et al. (1976) summarized the results of 14 other authors studying the origin of the extra chromosome 21 in Down syndrome. Sixty-two cases were informative out of the more than 243 studied. When these results were pooled, non-disjunction was found significantly more frequently in oogenesis than spermatogenesis. He also concluded that non-disjunction was much more common in meiosis I than meiosis II.

It was the intent of this study to determine if possible the origin of the extra D or G chromosomes in trisomic spontaneous abortuses.

MATERIALS AND METHODS

The spontaneous abortuses used in this study represent part of a larger series obtained by Dr. D.H. Carr from 1972-1976. Spontaneous abortuses were collected mainly from the Henderson General and St. Joseph's Hospitals in Hamilton, Ontario. A few were obtained from the McMaster University Medical Centre. The specimens were kept in sterile glass jars containing physiological saline. Specimens were selected for culture according to one of the following criteria: a ruptured or intact sac with loss of embryonic material, a structurally abnormal embryo, a macerated embryo, or hydatidiform degeneration of the chorionic villi. Abortuses possessing these features have been shown to have an increased risk of chromosome abnormality. Specimens were cultured according to the method previously described in detail (Carr, 1975). Briefly, this involved growing small pieces of embryonic membrane in culture. Usually chorion was chosen except where amnion and chorion could not be separated, in which case both were cultured. Fibroblasts were incubated until sufficient cellular proliferation had occurred. Cells were then harvested and a karyotype analysis performed. All cultures of abortus tissue and karyotype preparation was done by M. Gedeon.

For the purpose of this study, specimens were chosen which had a triploid karyotype or were trisomic for a chromosome in the D (13, 14, 15) or G (21, 22) groups. In total there were 20 triploids and 18 trisomies potentially available for study. Two additional triploids from the same time period were studied previously (McConnell and Carr, 1975).

The family physicians or obstetricians of the "mothers"* of these abortuses were contacted by Dr. Carr. The project was explained to them and they advised us as to whether or not the couple should be approached. There were several reasons why certain couples were deemed unapproachable. These included divorce or serious illness. Several couples had moved from the Hamilton area but we did manage to contact two of these. In total 29 couples were contacted. Of these, 14 were the parents of a triploid abortus and 14 of a D or G trisomy abortus. In addition, blood samples were provided by Dr. I. Uchida from the parents of a 68,XX abortus. Fibroblasts of the abortus were also provided. The study was explained to the

*A parent has been defined by Webster's Third International Dictionary as "the source from which something is derived". Therefore couples who produce an abortus rather than a live child can still be called parents and will be referred to as such.

couples by telephone. No couple to whom the purpose was explained refused to participate. Because of the family situation, one couple was not pursued after the initial telephone call. Arrangements were made with the remaining 28 couples, to obtain a venous blood sample. If possible the couples came to the McMaster Medical Centre and the blood was taken by Dr. Carr. If this was not convenient, Dr. Carr arranged to visit them at their home.

Twenty ml of blood were aspirated using a sterile heparinized glass syringe. The blood was immediately placed in a centrifuge tube containing 0.5 ml Hepalean (1000 heparin units per ml). Centrifuge tubes were stoppered and allowed to stand at room temperature for 3 hours. Approximately 2 ml of plasma were placed in an 84 ml medium bottle containing 10 ml of Connaught H597 culture medium. The pH of the medium was adjusted to 7.6-7.7 using sodium bicarbonate. Each culture also contained 0.1 ml of phytohaemagglutinin (Wellcome). Two cultures were prepared for each person. One contained a drop of packed red blood cells in addition to the plasma and one contained 2 ml of human AB serum (Hamilton Red Cross). Cultures were incubated at 37° for 3 days, then 0.1 ml colcemid (0.4 mg colchicine per ml) was added to each culture. After $2\frac{1}{2}$ hours in colcemid, cultures were transferred to centrifuge tubes and spun at 500 rpm for

6 minutes. The supernatant was discarded and approximately 5 ml of 0.075 M KCl was added to the pellet. Cells were kept in this hypotonic solution for 9 minutes and then centrifuged at 500 rpm for 6 minutes. The supernatant was again discarded and the cells fixed in 5 ml of fresh 3:1 methanol:glacial acetic acid. The tubes were stoppered and refrigerated overnight. The following day the cells were centrifuged at 500 rpm for 6 minutes, the supernatant discarded and the cells resuspended in a small amount of fresh fixative, just enough to make a milky suspension. Slides were prepared by dropping cells from a height of approximately 20 cm onto slides which had been presoaked in ice-cold water so that they were covered by a thin film of water. Slides were then air-dried by shaking vigorously over an alcohol lamp. Prepared slides were stored until needed.

Slides were stained for 2 minutes with quinacrine dihydrochloride made by dissolving 3 gm of Atebrin (G.T. Gurr) in 45 cc acetic acid and 55 cc distilled water. After staining, the slides were rinsed in 3 washes of distilled water. Coverslips were mounted in distilled water and sealed with wax. Cells were then observed with a Zeiss photomicroscope equipped with an HBO $200^{W}/4$ super-pressure mercury lamp. Exciter filters BG 12 and BG 38 and barrier filter 47 were in position. Slides were scanned

with the Planapochromat 40X, 1.0 N.A.; oil objective. Photographs of appropriate cells were taken using the Planapochromat 100X, 1.3 N.A., oil objective. Kodak high contrast copy film (ASA 64) was used with an average exposure time of 5 minutes. Films were developed using Kodak D-19 developer. Prints were made on F-2 or F-3 Kodabromide paper using Kodak Dektol developer and rapid fixer.

Approximately 40 photographs were taken of the chromosomes of each person. Chromosomes were identified according to the standards of the Paris Conference (1971). Satellite regions were described as being either non-existent (0), present (+), or intensely fluorescent (++). Centromeric bands on chromosomes 3 and 4 were either intense (+) or dull (-) and with chromosome 3 the position of the band was noted. After making observations on the polymorphic regions of the chromosomes of the parents and their abortus a comparison was made in order to try to determine which parent donated the extra chromosome or set of chromosomes and to determine, if possible, in which meiotic division the error occurred.

RESULTS

The characteristic banding patterns produced when chromosomes were stained with quinacrine dihydrochloride are illustrated in Fig. 2. A number of chromosomes exhibiting polymorphisms were used in determining the origin of the extra chromosome or set of chromosomes in trisomies and triploids. These are shown in Fig. 3.

Triploids: Four triploids were 69,XXX, ten were 69,XXY and one was 68,XX. All cultures of parents of triploid spontaneous abortuses were successful. The chromosomes of all parents were normal by the Q-banding technique.

Triploid No. 1 (69,XXX): Chromosome 22 was used as the marker chromosome in this abortus (Fig. 4). The triploid had two 22's with bright satellites and one non-bright. The father had one 22 with bright satellites, whereas both of the mother's 22's were not bright. This indicated either failure during meiosis II of spermatogenesis or dispermy.

Triploid No. 2 (69,XXX): It can be seen in Fig. 4 that the father of this triploid had one chromosome 15 with bright satellites and one with non-bright satellites.

The mother's 15's were not satellited. The abortus had two 15's with bright satellites and one without visible satellites. Again, the triploid could have arisen by failure during meiosis II or by dispermy.

Figure 2: Q-banded karyotype of a normal 46,XY male


X Y

Figure 3: Polymorphic features of chromosomes 3, 4, 9, 13, 14, 15, 21 and 22 which were used as markers

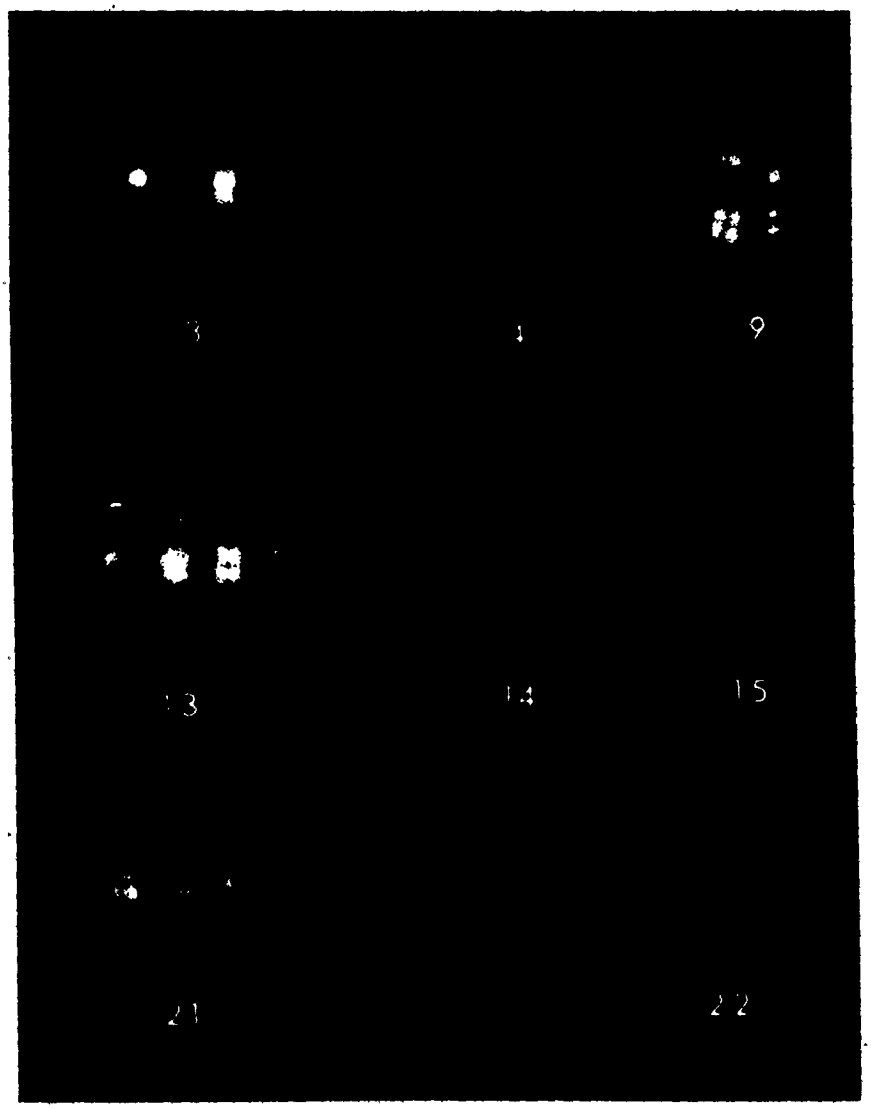
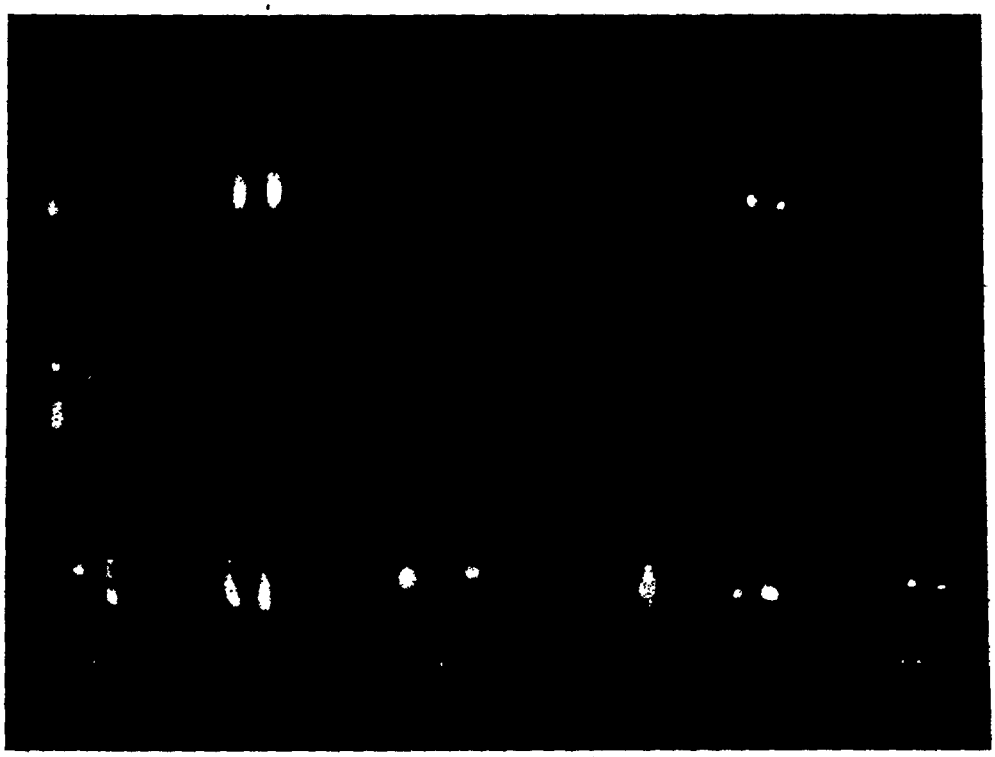
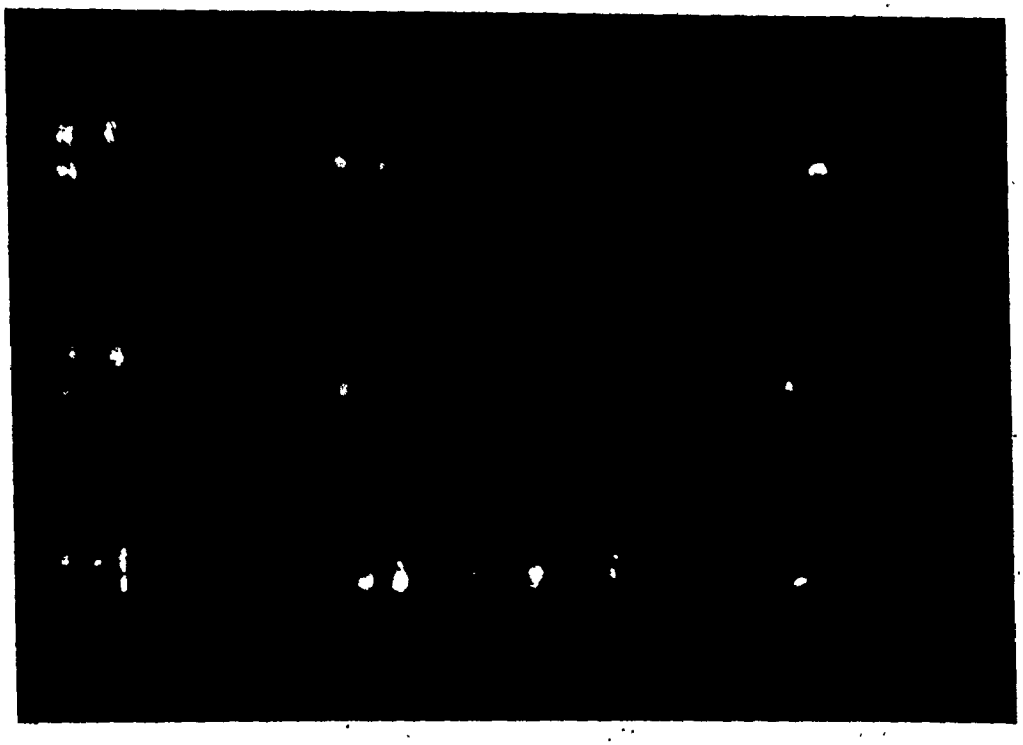

3
4
9
13
14
15
21
22

Figure 4: Top - Marker chromosomes of parents and triploid abortus No. 1 (69,XXX). Chromosome 22 was used as the marker. Triploid inherited the extra chromosomes from the father.

Bottom - Some chromosomes of parents and triploid No. 2 (69,XXX) in which chromosome 15 was the informative marker and showed that the extra chromosomes were donated by the father either by failure of meiosis II or dispermy.





Triploid No. 3 (69,XXX): Some chromosomes of this triploid and the parents are illustrated in Fig. 5. Chromosome 13 was used as the marker. The mother's 13's contained no bright polymorphic regions, whereas the father had one chromosome 13 with bright satellites and one with non-bright satellites. The abortus had two 13's with bright satellites which were identical to one in the father. This triploid arose by failure during meiosis II or dispermy.

Triploid No. 4 (69,XXX): Both chromosomes 3 and 4 were used in determining the origin of this triploid, illustrated in Fig. 5. The abortus had two 3's with a bright fluorescent band on the long arm and two 4's with bright bands at the centromere. Only the mother's chromosomes possessed these two features. Because the two maternal 3's in the abortus were identical yet the mother had one 3 with no fluorescent band near the centromere, then the failure must have occurred during meiosis II of oogenesis.

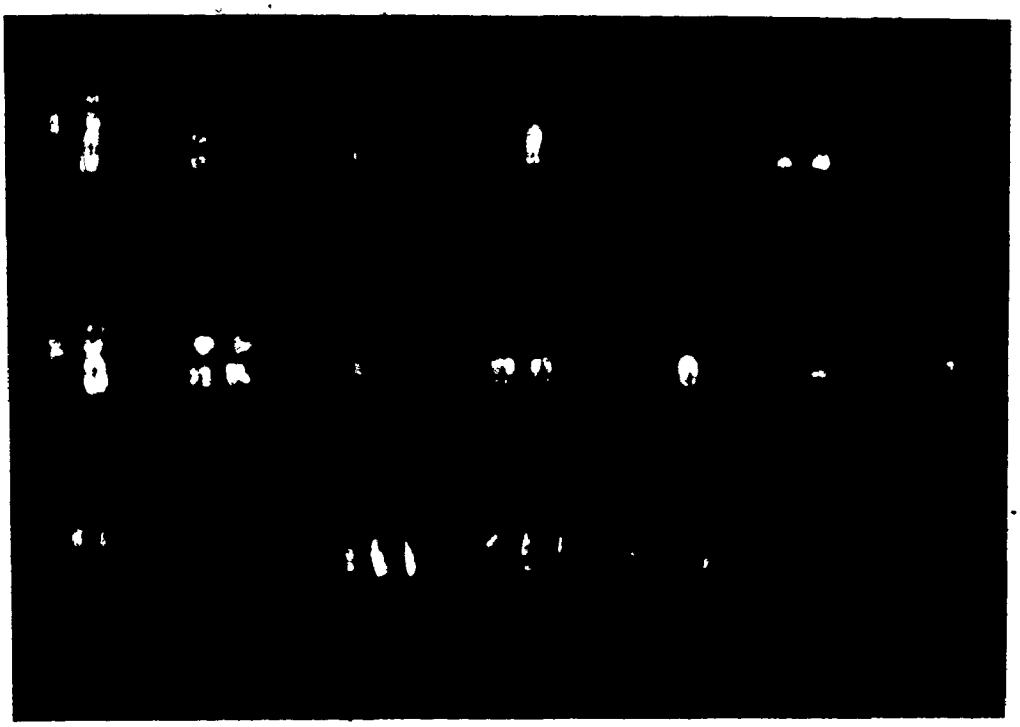
Triploid No. 5 (69,XXY): Chromosome 4 was used as the marker in this triploid (Fig. 6). The triploid had two 4's with a fluorescent centromeric band. The father had one 4 with this same band but his other 4 and both 4's of the mother lacked this band. The extra haploid set of chromosomes was donated by the father either

by dispermy or failure of meiosis II. However, the paternal sex chromosomes were XY, which could not occur from failure of meiosis II. Dispermy was the mechanism for the origin of this triploid.

Triploid No. 6 (68,XX): This abortus shown in Fig. 6 had two 14's with bright satellites and one with no satellites. Only the mother had a chromosome 14 with bright satellites. The error occurred during meiosis II of oogenesis.

Figure 5: Top - Chromosome 13 was used as the marker in triploid No. 3 (69,XXX). The father donated the extra 23 chromosomes.

Bottom - Triploid No. 4 (69,XXX) resulted from failure of meiosis II of oogenesis. Chromosome 3 was the informative marker.



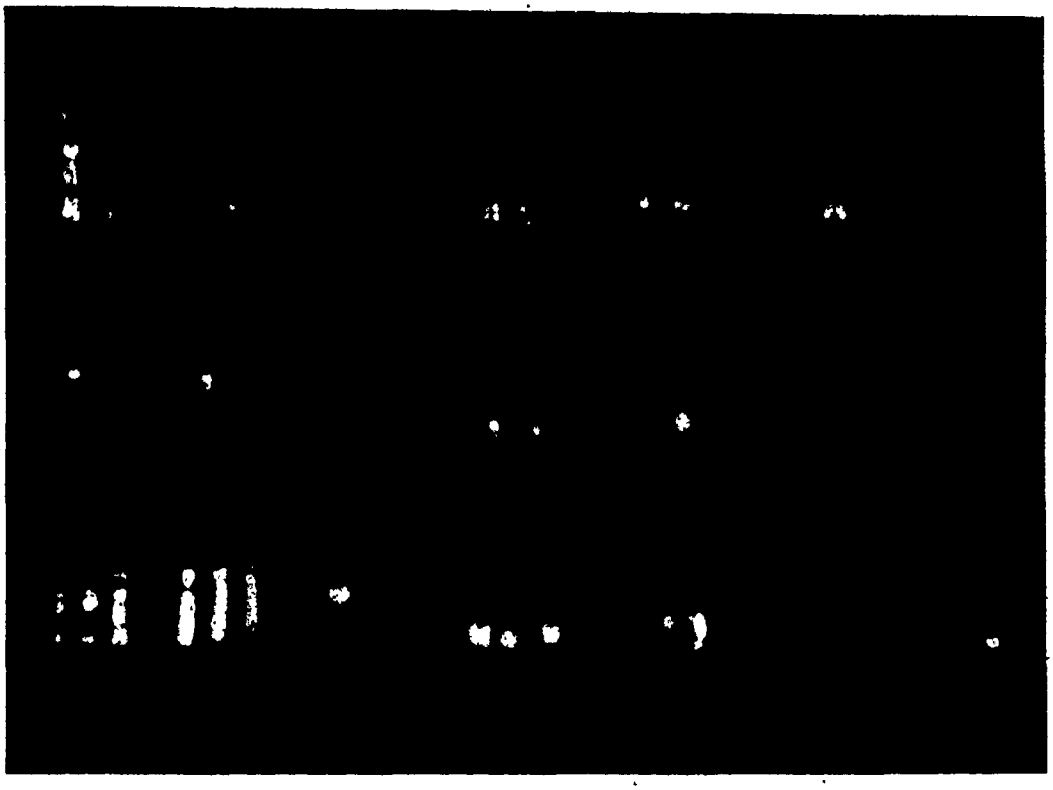
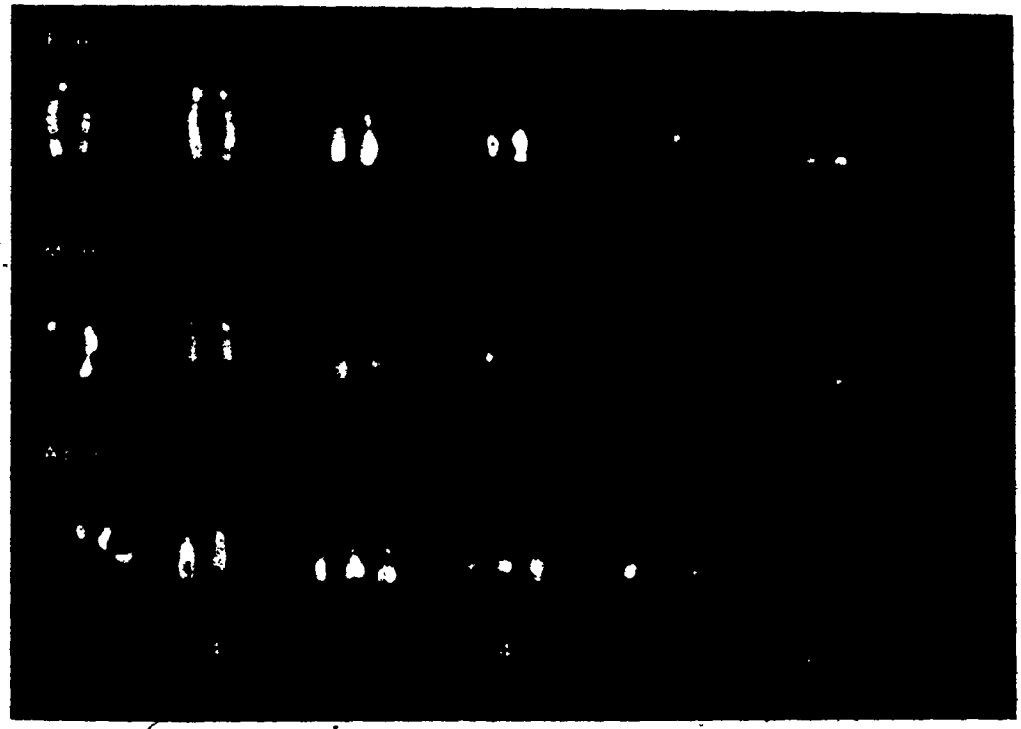
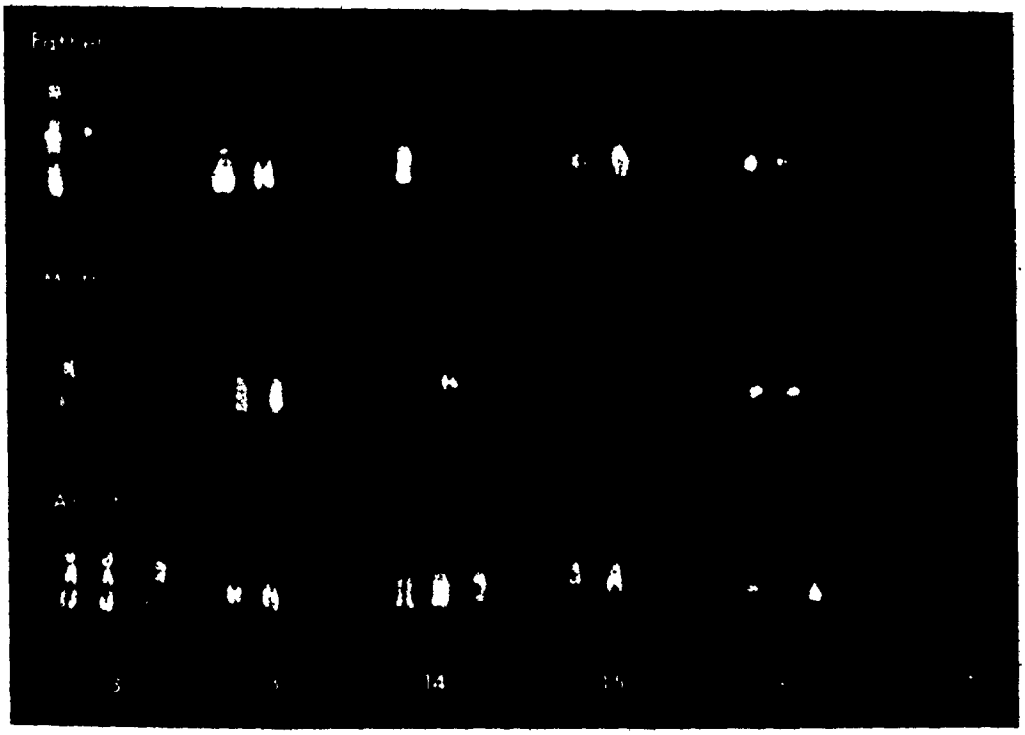


Figure 6: Top - Triploid No. 5 (69,XXY) arose by dispermy. The abortus had two chromosome 4's with fluorescent centromeric bands. Only the father possessed a 4 with the band.

Bottom - Chromosome 14 was the informative marker in triploid No. 6 (68,XX). The conceptus arose by failure of meiosis II of oogenesis.



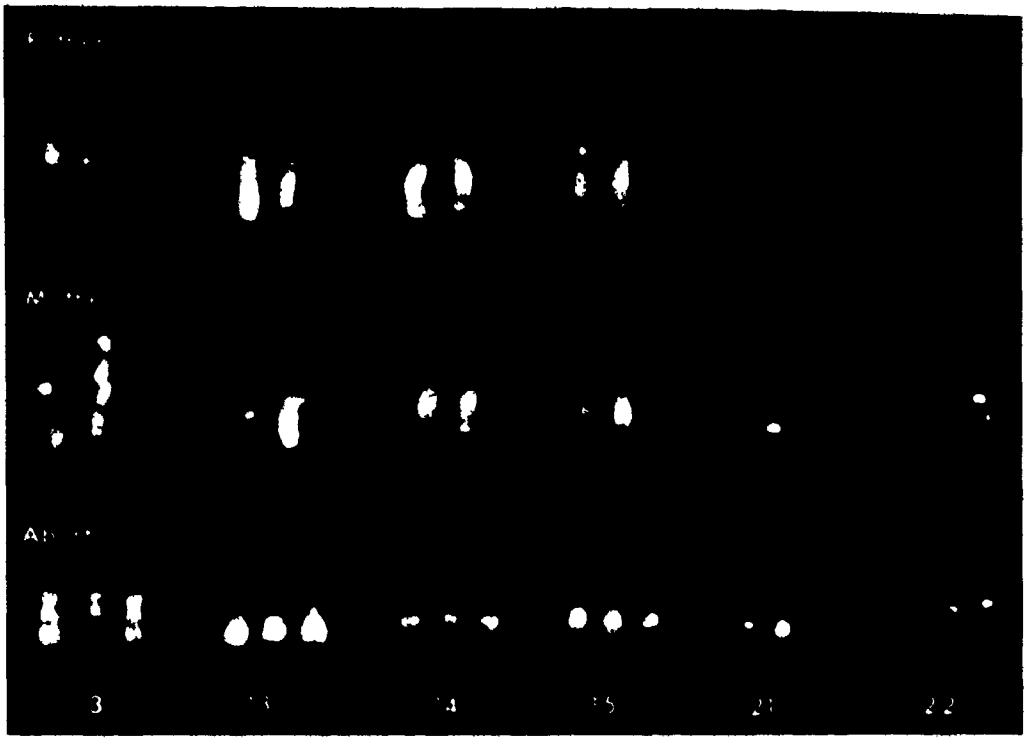


Triploid No. 7 (69,XXY): The chromosomes of this triploid and the parents are illustrated in Fig. 7. Chromosome 22 was used as the marker. The abortus had two 22's with brightly fluorescent satellites and one with no brightness. Only the mother had a 22 with such a brightly fluorescent area. The triploid must have arisen from a haploid sperm and a diploid ovum, which resulted from failure during meiosis II of oogenesis.

Triploids 8-15 (all 69,XXY): The origin of the extra set of chromosomes in these triploids was impossible to determine. The chromosomes of these triploids and their parents are shown in Fig. 7-11. There was no suitable marker which allowed unequivocable determination of their origin. However, there are several possibilities for their origin which can be proposed. This was done by choosing one chromosome as a marker and then ruling out unlikely possibilities. Several alternatives remained. These are illustrated in Table 1.

Figure 7: Top - Triploid No. 7 (69,XXY) resulted from failure of meiosis II of oogenesis. Chromosome 22 was the informative marker.

Bottom - Some marker chromosomes of parents and triploid No. 8 (69,XXY). There was no informative chromosome marker.



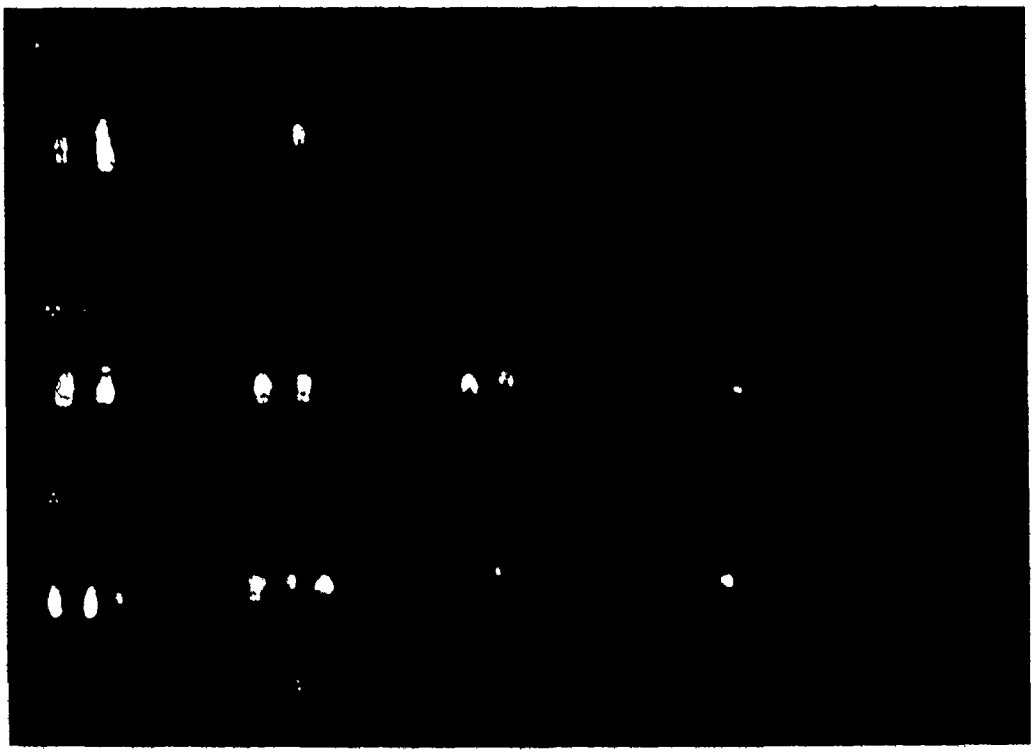


Figure 8: Top - Some marker chromosomes of triploid No. 9 (69,XXY) and the parents. There was no informative marker.

Bottom - Some marker chromosomes of the parents and triploid No. 10 (69,XXY). The parental origin of the triploid could not be determined.

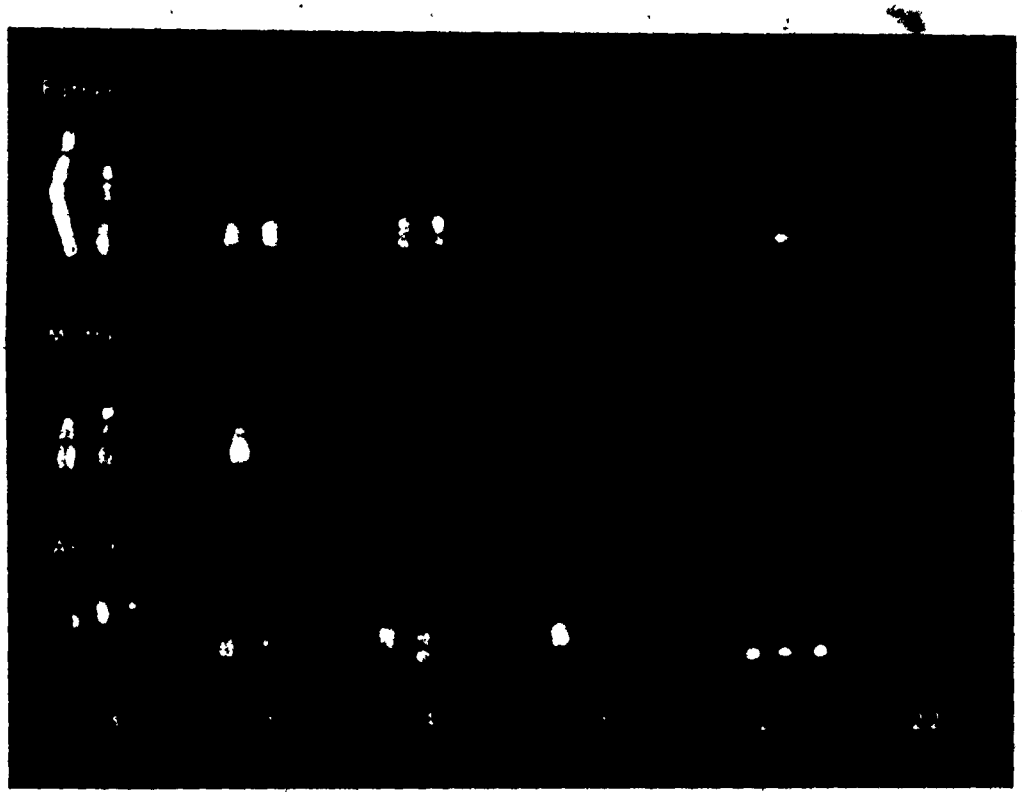
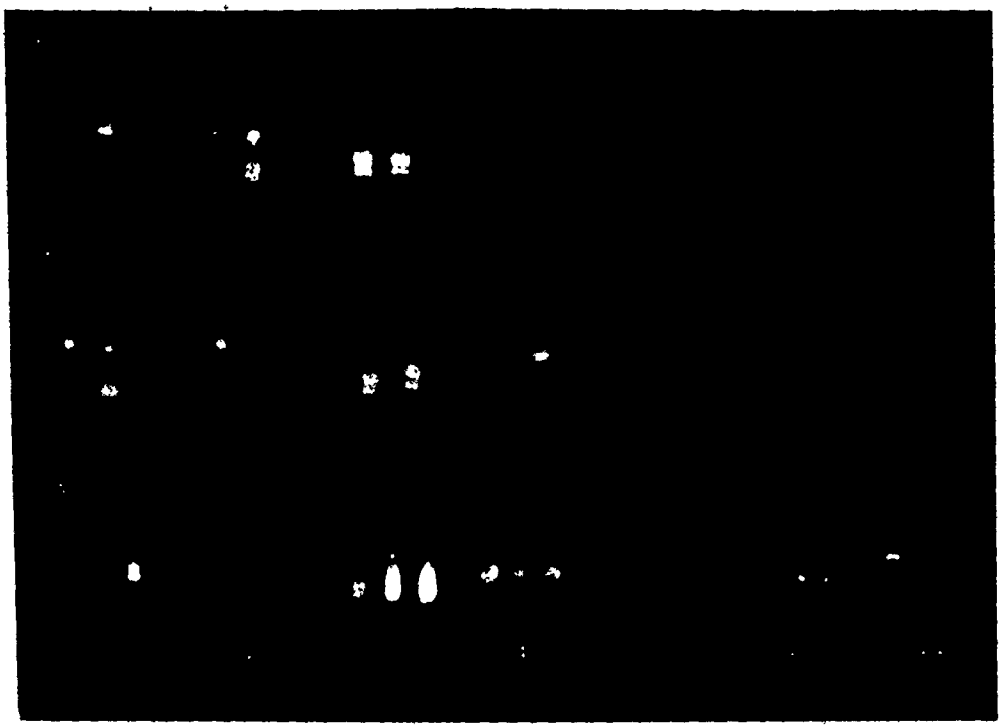


Figure 9: Top - Some marker chromosomes of triploid No. 11 (69,XXY) and the parents. There was no informative marker.

Bottom - Some marker chromosomes of the parents and triploid abortus No. 12 (69,XXY). There was no informative marker.



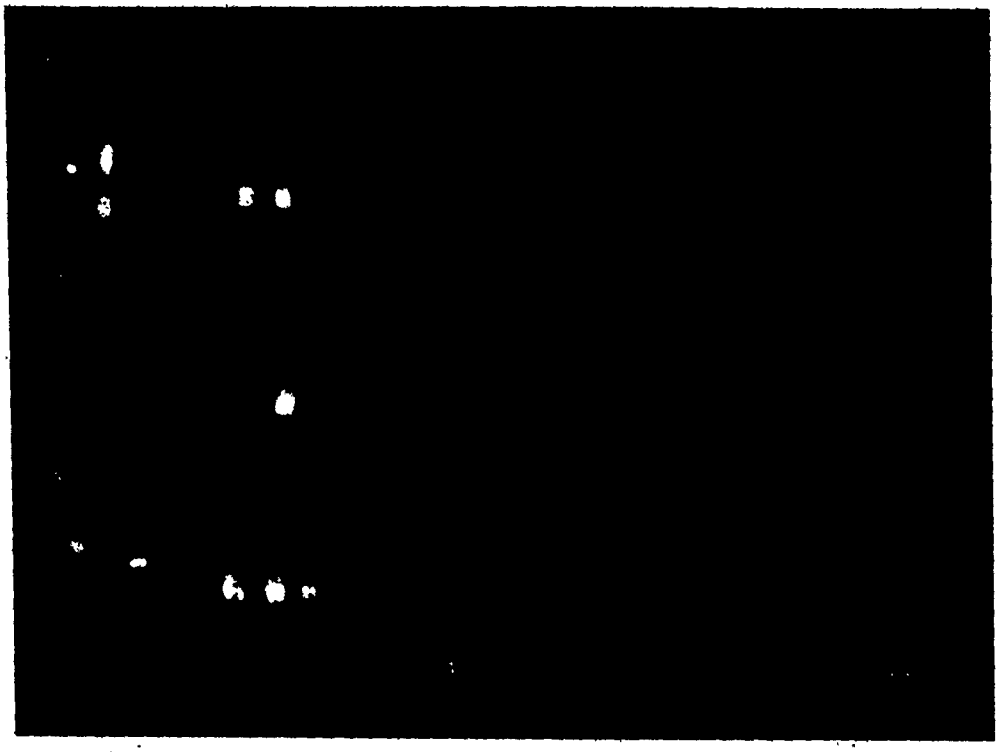


Figure 10: Top - Some marker chromosomes of triploid abortus No. 13 (69,XXY) and the parents. The parental origin of the abortus could not be determined.

Bottom - Some marker chromosomes of triploid No. 14 (69,XXY) and the parents. There was no informative marker.

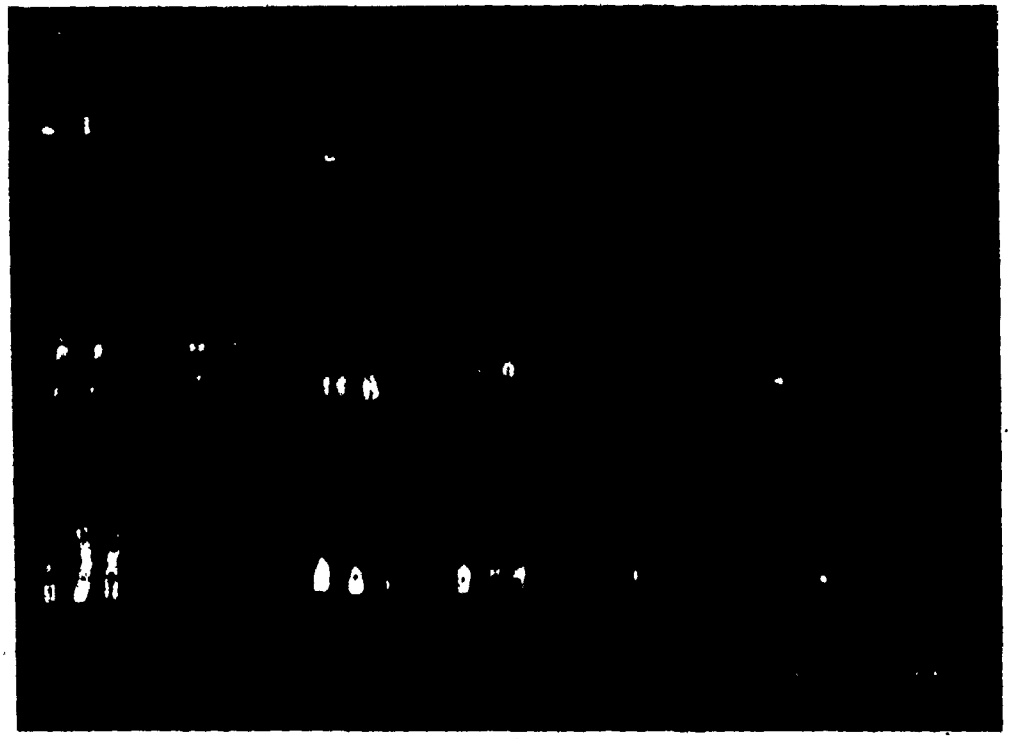


Figure 11: Some marker chromosomes of triploid abortus No. 15 (69,XXY) and the parents. There was no informative marker so the parental origin of the triploid is not known.

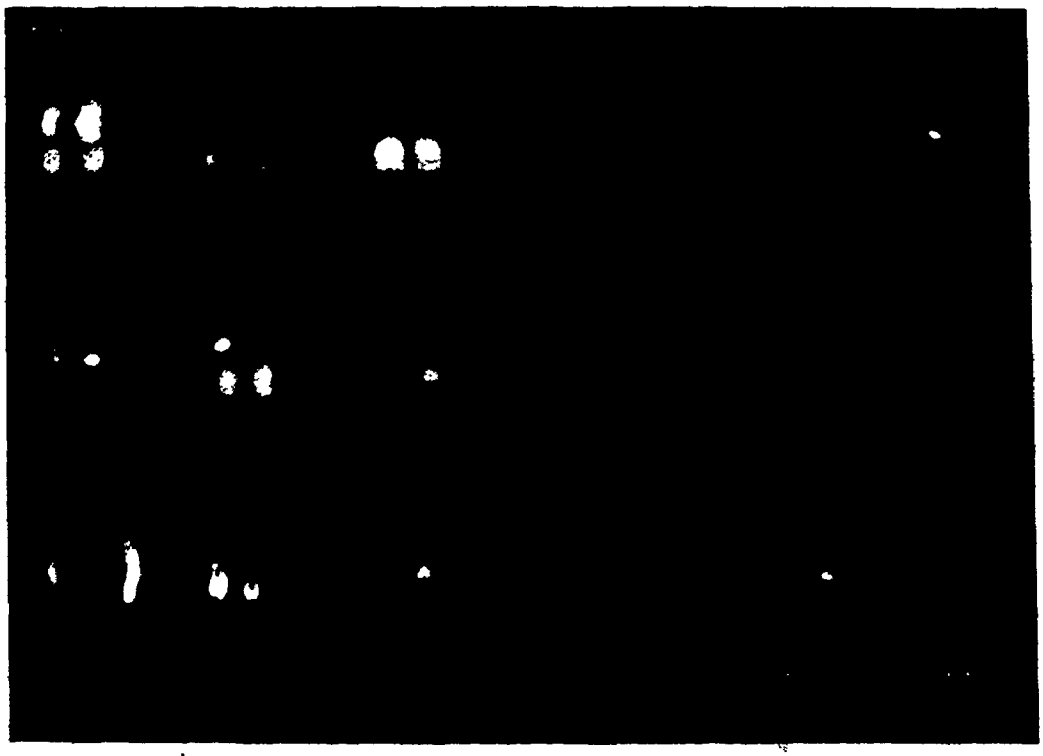


Table 1: Illustrates the probable origin of the fifteen triploid spontaneous abortuses

Triploid No.	Oogenesis		Spermato-genesis		Dispermy	Chromosome Marker
	MI	MII	MI	MII		
1 XXX				+	+	22
2 XXX				+	+	15
3 XXX				+	+	13
4 XXX		+				3,4
5 XXY					+	4
6 XX		+				14
7 XXY		+				22
8 XXY	+	-	+	-	+	21
9 XXY	+	-	-	-	+	15
10 XXY	+	+	-	-	+	15
11 XXY	+	-	-	-	+	14
12 XXY	-	+	-	-	+	14
13 XXY	+	+	+	-	+	14
14 XXY	-	+	+	-	+	14
15 XXY	+	+	+	-	+	22

+ Above event is likely

- Above event is unlikely

Trisomies: Of the trisomies examined four were trisomy 13, one was trisomy 14, one trisomy 15, three were trisomy 21 and three were trisomy 22. In addition, the cultures of parents of a trisomy 21 were unsuccessful and in a trisomy 14 the father refused to give a blood sample although the mother was willing. The chromosomes of the mother alone were not informative. Of the remaining 12 trisomies studied the parental origin of only one could be determined. This was the first trisomy 22 illustrated in Fig. 13. Only the mother had brightly satellited 22's. The abortus had two 22's with bright satellites. It was not possible to determine whether the non-disjunction occurred during meiosis I or meiosis II. All other trisomies were uninformative because of the similarity of parental markers.

Figure 12: Abortuses possessing D group trisomies and the corresponding chromosomes of the parents are shown. There were four trisomy 13's, one trisomy 14 and one trisomy 15. The parental origin of none of these trisomic abortuses could be determined because of the similarity of parental markers.

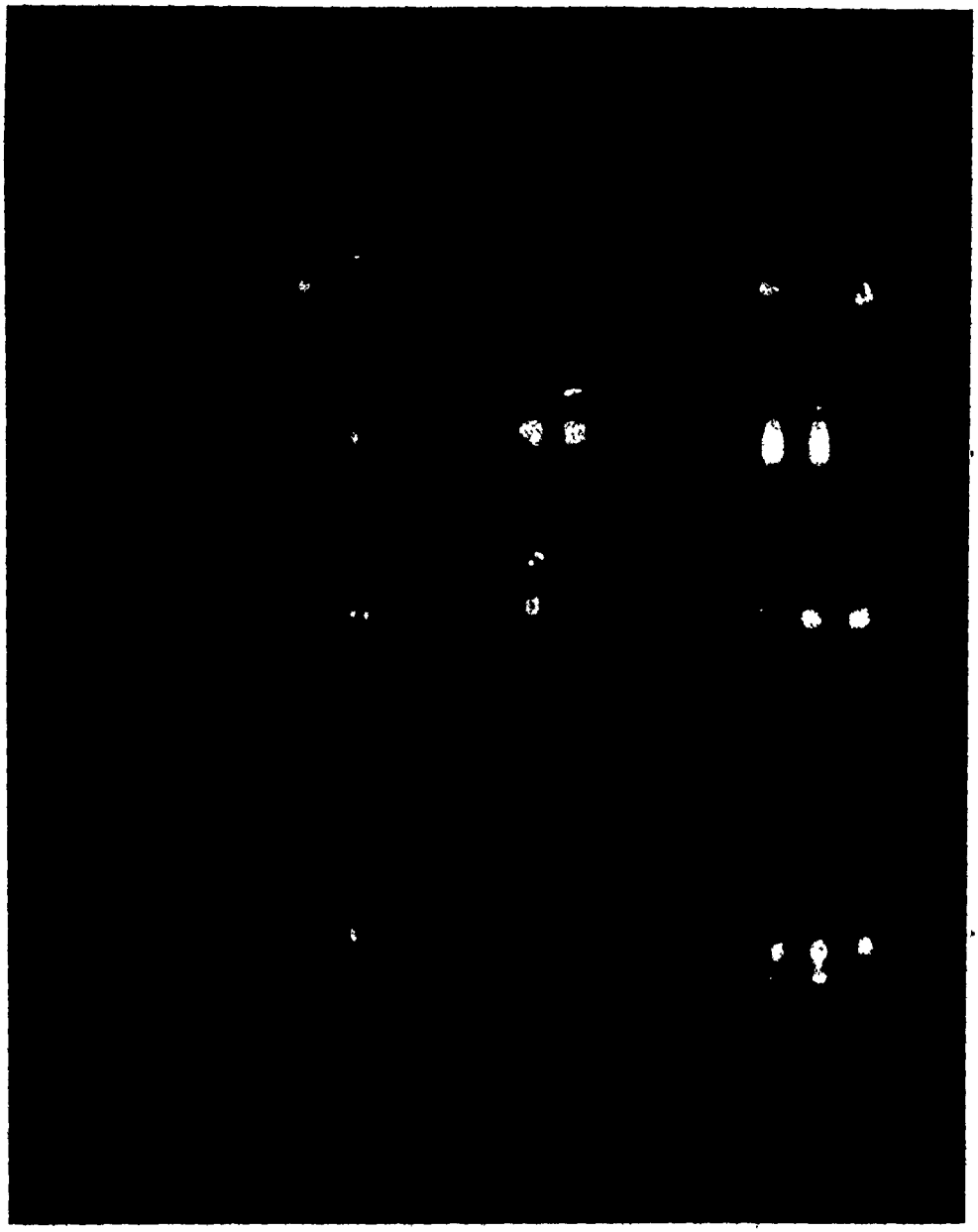
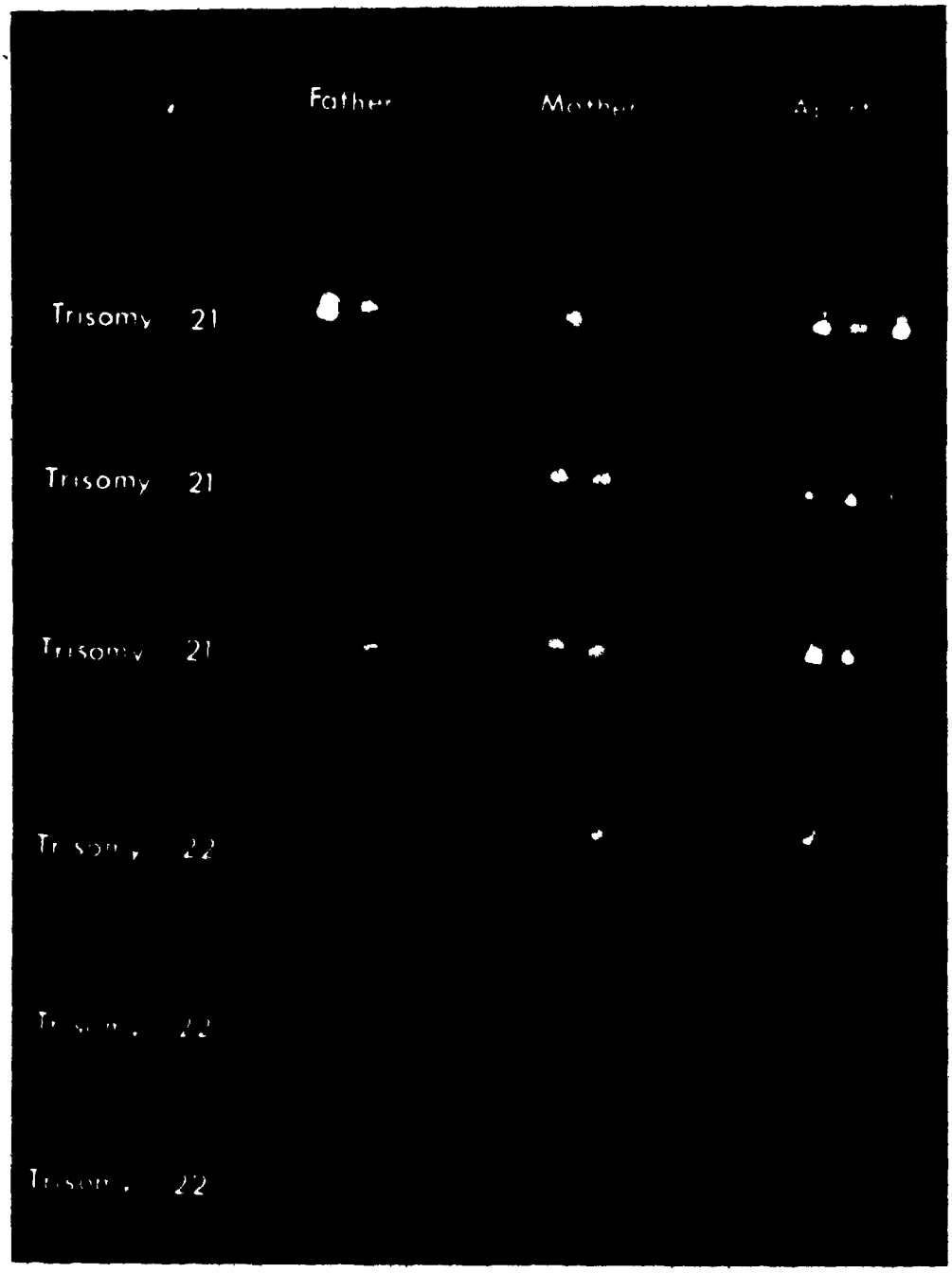


Figure 13: The trisomic chromosomes of abortuses possessing G group trisomies and the corresponding chromosomes of the parents are shown. There were three trisomy 21's and three trisomy 22's. The origin of only one of these could be determined. This is the first trisomy 22 shown in this figure. The extra chromosome came from the mother although in which meiotic division the non-disjunction occurred was not clear.


Father
Trisomy 21
Trisomy 21
Trisomy 21
Trisomy 22

DISCUSSION

Human chromosome polymorphisms have proved useful in the determination of the parental origin of the extra chromosomes found in D and G trisomies and triploids. However, the polymorphic fluorescent regions, especially the satellites, have certain limitations which must be recognized. For example, satellites may be bent under the chromosome and thus appear to be missing. For this reason analysis should not be made from only one cell. Another factor is the intensity of fluorescence in a photograph. This depends on many factors, including the intensity of the negative and the exposure time of the print. Any overlapped chromosomes will appear much brighter than if they were separate. For these reasons initial scoring of variants is best made directly from the slide. Realizing the limitations of fluorescence polymorphisms it was decided not to employ the five-level scale of fluorescent intensity suggested at the Paris Conference. A simplified scheme consisting of three categories was used. These categories are absence of brightness, fluorescence present but not brilliant and brilliant fluorescence. This was believed to be more objective than the five-level scale since it eliminated

the need to discriminate between very similar levels of intensity.

Overton et al. (1976) reported great success in determining the origin of the extra chromosome 21 in Down syndrome. Their success stemmed from darkroom techniques in which they made serial prints of each 21 using increments of 1 second exposure time, from underexposure to over-exposure. They believed that the number of exposures necessary to produce visible satellites could be used as a heritable characteristic. Because of the number of variables involved such a conclusion is not justified.

Origin of Triploidy

Of the 7 triploids which were informative there were almost equal numbers in which the extra chromosomes were from the mother (3) and the father (4). These 7 cases represent 47% of the triploids examined. The increased number of informative cases compared to the autosomal trisomies is due to the fact that more chromosome pairs are available for study. Because triploids possess a complete extra haploid set of chromosomes, a polymorphism on any chromosome may be useful as a marker. It is interesting to note that in the 3 cases in which the extra chromosomes were from the mother, the triploids were due to an error in the second meiotic division. In

3 of the 4 triploids in which the extra chromosomes were paternally derived, it was not possible to distinguish between non-reduction during meiosis II or fertilization by two sperms. The fourth case definitely arose by dispermy.

Origin of D, G Trisomies

The fact that a very small number of autosomal trisomies (1 out of 12 examined) was informative as to the origin of the extra chromosome is in agreement with other authors. Informative cases are those in which it is possible to trace each marker in the abortus to one parent unequivocably. In order to do this there must be differences in the parental markers for the chromosome under consideration. If both parents are homozygous or heterozygous for the same marker then it is not possible to determine which parent donated the extra chromosome. The reason why the number of informative cases in autosomal trisomies is so small is because only one chromosome pair is available for study. Certain polymorphic features are much more common than others so similar patterns for a couple are more probable than dissimilar ones. For example, Muller et al. (1975) reported that 97.5% of chromosome 14's have no brightly fluorescent band either on the short arm or in the centromere region. Therefore, the probability of one member of the couple having such a band is much

smaller than the probability that neither will have it. If one member of the couple did possess such a less frequent characteristic then there is a good chance it would be useful as a marker.

Robinson and Newton (1977) examined the chromosomes of Down syndrome individuals. They defined positive satellites as those which were brilliantly fluorescent and normal satellites were those which contained no quinacrine fluorescence or the fluorescence was not intense. They found that the incidence of positive satellites on chromosome 21 in Down syndrome was significantly greater than controls. They hypothesized that this may indicate an association between this polymorphism and the tendency to non-disjunction. If this were true, the probability of a woman producing a Down syndrome child could be based on more than just her age. More data need to be collected by other authors to verify this is a true association and in addition the parents of Down syndrome individuals would be expected to have an increased frequency of positive satellites over controls. This also needs to be examined. Robinson and Newton (1977) also found that 21's with positive satellites associate less often than those with normal satellites. Originally it was proposed that there was a link between satellite association of chromosome 21 and non-disjunction (Evans, 1967). This

could also be related to the positive satellites just mentioned. However, Taysi (1975) examined the incidence of satellite association in parents of Down syndrome individuals and controls. He found that for both groups chromosome 21 was more frequently involved in associations than other acrocentrics. There was no difference between the groups indicating that satellite association does not seem to be related to non-disjunction directly. Therefore, if the presence of positive satellites on chromosome 21 affects its behaviour in such a way as to increase its rate of non-disjunction, then it must act through a different mechanism than by satellite association.

It is generally agreed that the extra chromosome in the maternal age-dependent category of D and G trisomies are inherited from the mother. It is the maternal age-independent trisomies in which paternal non-disjunction must occur (Mikkelsen et al., 1976). Indeed, non-disjunction during spermatogenesis leading to D and G trisomies, especially 21, is much more common than originally thought. Langenbeck (1976) reviewed the studies on the origin of the extra chromosome in trisomy 21. In 31% of informative families the non-disjunction occurred in the father. He also concluded that non-disjunction during meiosis I was much more common than in meiosis II.

The only trisomy which was informative in this study was a trisomy 22 in which the extra chromosome was definitely from the mother. However, it was not possible to distinguish between non-disjunction during meiosis I and meiosis II. The maternal age at the time of this abortion was 39 years.

Causes of Triploidy and Trisomy

Aging of gametes has been proposed as the major cause of chromosome anomalies which are found in such great numbers in spontaneous abortions. This aging does not have to coincide with chronological aging of one or both parents. Aging of oocytes may be of two types: intrafollicular, in which the ovum is retained within the follicle longer than the normal time; and intratubal (post-ovulatory overripeness) in which ovulation occurs at the normal time but there is a delay before fertilization. Aging of sperm may occur within either the male or the female reproductive tract. Human sexual behaviour is such that it offers no protection against aging of gametes. Sexual activity is not restricted to a narrow time period around ovulation as it is in animals that have a specific estrous period, such as dogs. Other animals, for example rabbits, have coitus-induced ovulation. Both of these mechanisms ensure that ova will not be aged before fertilization and that sperm will not be aged before they

contact the ova. In humans because sexual activity may occur at any time during the monthly cycle there is the potential for both types of gametes to be aged before fertilization.

A number of studies have been carried out, mainly in animals and occasionally in man, to examine the specific effects of aging of gametes on zygotic development. Fugo and Butcher (1966) delayed ovulation in rats for 48 hours. They found that polyspermy was three times more frequent with delayed ovulation than with controls. They also studied the chromosomes of the zygotes which resulted from delayed ovulation. They found a wide range of chromosome anomalies similar to those found in humans. Mikamo (1968) studied the effects of intrafollicular overripeness on the development of Xenopus embryos. Frogs were prevented from ovulating for extended periods and then ovulation was induced. Abnormal movement of undivided tetrads or dyads to spindle poles was observed. Shaver and Carr (1967) found that triploidy was the most common anomaly when there was a delay of 4 - 5 hours between actual ovulation and fertilization in the rabbit. Vickers (1969) also caused a delay between ovulation and fertilization in mice, for 7 - 13 hours, and found a nine-fold increase in triploid embryos compared with controls.

In a study involving humans, Guerrero and Rojas (1975) examined spontaneous abortion and its relation to the time of fertilization. Women were following their basal body temperature as an indication of ovulation and reported when coitus had occurred. They found that when sperms were aged before fertilization or when there was a delay between ovulation and fertilization there was an increased risk of spontaneous abortion. The lowest rate of such abortions occurred in a narrow time period around ovulation. If spontaneous abortions can be used as an indicator of chromosome anomalies (since 50% of spontaneous abortions have such an anomaly) then aging of either the ovum or the sperm may cause an increased risk of chromosome abnormalities in humans.

Sperm may be aged in the male reproductive tract as well as in the female. In humans this results from periods of sexual inactivity. Little is known about this type of aging of sperm in humans. Martin-Deleon et al. (1973) examined the effects of sperm aged in the male reproductive tract in rabbits. They found that when sperm were aged for 1 - 3 weeks a number of abnormalities were found in the zygotes, the most common of which was autosomal trisomy. Fechheimer and Beatty (1974) presented evidence that, at least in rabbits, diploid sperm are unlikely to give rise to triploid zygotes. Semen containing 0.4, 1.5

and 2.9% diploid sperm was used for artificial insemination. They found no increase in the incidence of triploidy with greater concentrations of diploid sperm.

The findings discussed thus far, if applicable to humans, offer explanations for the results obtained with triploid abortuses. In 3 of the 4 triploids in which the extra chromosomes were derived from the father it was not possible to distinguish between failure of meiosis II and dispermy. From the animal data, dispermy is a more probable mechanism than fertilization with a diploid sperm. In order for dispermy to occur there must be a breakdown of the barrier which inhibits entry of more than one sperm. Normally after one sperm penetrates the zona pellucida there are changes in this layer such that it becomes impermeable to additional sperm (Austin, 1970). The work of Fugo and Butcher (1966) showed that, in mice, aging of an ovum causes changes in the zona pellucida such that more than one sperm can penetrate the ovum. In all 3 triploids in which the extra chromosomes were maternal, the conceptus arose by failure to extrude the second polar body. There is evidence that delaying fertilization, in addition to increasing the rate of dispermy, also causes retention of the second polar body (Braden and Austin, 1954). Szollosi (1973) described changes in the meiotic spindle with post-ovulatory aging

of the ovum which are probably responsible for the failure to extrude the second polar body. Since the incidence of triploidy does not increase with advanced maternal age, the aging of the ovum which is responsible for the production of a triploid zygote is probably post-ovulatory overripeness. This is supported by the finding that meiotic errors in the mother occurred during meiosis II. Lauritsen (1976) reported two triploids in which the error occurred in meiosis I in the mother. Therefore, pre-ovulatory aging must be involved in some cases.

Intrafollicular overripeness is one of the possible mechanisms for the production of D and G trisomies, especially those which exhibit maternal age dependence. This type of aging may affect either meiotic division as shown by Mikamo (1968). There are a number of other possible etiological factors to account for those trisomies which are not related to late maternal age and those in which the extra chromosome is from the father. One which has been well-established is maternal irradiation. Uchida et al. (1968) found that women who underwent abdominal radiation prior to their pregnancy were more likely to produce trisomic offspring than controls. The effect was more pronounced in the late reproductive years of the women. Uchida and Freeman (1977) found experimental evidence in mice of a similar phenomenon. They

found that the sensitivity of mice to radiation increased with age. Kochupillai et al. (1976) studied a population in Kerala, South India which has a high level of background radiation. They found that the incidence of Down syndrome was significantly greater than in a nearby control population which had a much lower level of background radiation. The populations were similar in other respects so it was considered that radiation was the only important variable. It thus appears well-documented that radiation whether used therapeutically or at high levels in the environment is capable of genetic damage, especially increasing the rate of non-disjunction.

Another factor which may affect the rate of non-disjunction is the presence of thyroid antibodies. Fialkow et al. (1965) found that the frequency of thyroid autoantibodies was greater in the mothers of Down syndrome than control mothers. These antibodies could either be the cause of the non-disjunction or be the result of carrying a fetus containing the non-disjunction chromosomes. At present this issue has not yet been resolved. Dallaire and Leboeuf (1973) presented evidence that the autoimmune process in the mothers of Down syndrome children was multi-organ specific and hypothesized that ovarian antibodies could lead to the disruption of normal meiotic events.

Epidemiological studies have shown the peak conception rates of Down offspring to occur in the months of May and November (Harlap, 1974). Janerich and Jacobson (1977) proposed an endocrinological theory to account for the seasonal fluctuation in the incidence of conception of Down syndrome. This is just a theory with no data to support it.

In some families there may be more than one incidence of Down syndrome which is not related to late maternal age and the chromosomes of the parents are normal. One mechanism for the production of repeated trisomies is gonadal mosaicism.

One other factor which may be causative in the production of trisomies is drugs. Some evidence suggests that certain drugs such as analgesics, antipyretics and antibiotics may cause an increase in non-disjunction if taken around the time of conception (Private communication to Dr. I.A. Uchida, from Dr. M. Yamamoto). The effect of drugs in causing non-disjunction is not well-documented at this time.

The relative importance and the interactions between these various factors in the production of trisomies is not known. There are probably additional agents which also are causative. The relationship of non-disjunction

to late maternal age is the one factor which has been well studied but any of the other factors may also be related to age, such as radiation.

At present, a very small proportion of autosomal trisomies and approximately 50% of triploid spontaneous abortions are informative as to the origin of the extra chromosomes when quinacrine fluorescence polymorphisms are employed. Table 2 outlines which chromosome markers were found useful for determining the parental origin of the extra chromosomes. There was no chromosomal marker which was found to be consistently of value. In most informative triploids there was only one definite marker. In 3 of the 4 informative diandric triploids it was not possible to distinguish between failure of a meiotic division and dispermy. Ideally to accomplish this, a polymorphism on each chromosome would be needed. In the future as more and more banding techniques are attempted such a dye or combination of stains may be found. Perhaps it will be possible to predict on the basis of an individual's (or couple's) chromosomes, the probability of spontaneous abortion or of a specific chromosomal defect.

Table 2: Illustrates which chromosome polymorphism was useful in determining the origin of the informative triploids.

Triploid Number	Chromosome Marker Used
1 XXX	22-bright satellites
2 XXX	15-bright satellites
3 XXX	13-bright satellites
4 XXX	3-bright band on long arm
5 XXY	4-fluorescent centromeric band
6 XX	14-bright satellites
7 XXY	22-bright satellites

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