KARYOTYPIC ANALYSES OF HUMAN PERIPHERAL LEUKOCYTES

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An Abstract

Presented to

the Graduate Council of

Austin Peay State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

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by

Reta Jane Hamilton

March 1971

Abstract

Karyotypic analyses of human peripheral blood leukocytes were made in order to identify any numerical or structural anomalies. Prepared slides of cultured leukocytes were photographed and examined for chromosomal abnormalities. Normal karyograms were constructed from two phenotypically abnormal patients. Three abnormal karyotypes were observed: two with Down's syndrome (Trisomy 21) and one cri-du-chat syndrome (deletion of a portion of the short arm of chromosome 5).

The parents of one of the patients with Down's syndrome were exceptionally young (23 and 21 years). Karyograms constructed from the father were normal. Analysis of the mother indicated the presence of two cell populations, one normal and one abnormal. In the latter population, two of the 46 chromosomes were unpaired. One of these is thought to be the X chromosome. The identity of the smaller chromosome is not known but resembles the shortest pair in the C group.

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March 1971

To the Graduate Council:

I am submitting herewith a Thesis written by Reta Jane Hamilton entitled "Karyotypic Analyses of Human Peripheral Leukocytes." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biology.

Daniel W. Bath, Jr.
Major Professor

We have read this thesis and recommend its acceptance:

Floyd M. Ford
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TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

Recent changes in cytological techniques have enabled researchers to study and identify human chromosomes. It was not until 1956 that Tjio and Levan found the correct human diploid chromosome number to be 46 which includes 44 autosomes and 2 sex chromosomes. The human chromosomal complement may be described as 46, XY for a male and that of a female as 46, XX. Ford and Hamerton (1956) confirmed the chromosome number of 46 and provided unequivocal evidence for the presence of an X- and a Y-chromosome in human spermatocytes. The sex chromosomes are recognized because of the unequal pair in the male, while in the female the two X-chromosomes are similar in appearance to the single X-chromosome of the male.

Two conferences have been convened to standardize the nomenclature of the human chromosomes. The Denver Conference in 1960 proposed that for the classification of human chromosomes in mitotic metaphase the autosomal pairs be serially numbered in descending order of size from 1 to 22 and the allosomes designated with XX or XY. An additional distinguishing marker is the centromeric position of each chromosome. This serial arrangement of chromosomes is known as a karyotype or karyogram (Mittwoch, 1967).

The Denver group met with considerable criticism and was later superseded by the London Report (1963). This

report is a much more cautious document and is not open to the objections leveled against the Denver Report. The human complement was divided into seven groups. A chromosome group is designated either by the numbers of the smallest and the largest chromosome occurring in it or by a capital letter; therefore, group 16-18 is synonymous with the E group. Identification of autosomes are designated by assigned numbers so that the smaller of the chromosome pairs has the larger number. There is rarely any doubt as to which group a given chromosome belongs, although it is sometimes difficult or impossible to identify individual chromosomes within each group (Yunis, 1965).

The analysis of human karyotype is essentially based on the fact that a chromosome may be defined by two parameters, its length and the position of its centromere. Cytological identification of individual chromosomes can be made by at least three different criteria: (1) by the lengths of their two arms with due allowance for differences in contraction, (2) by secondary constrictions, and (3) by autoradiographic labeling patterns. Individual chromosomes are characterized by the length of their two arms or by their total length and arm ratio. The arm ratio is the length of the long arm divided by that of the short one. The centromere index is sometimes used, which is the length of the short arm divided by the total chromosome length, instead of the arm ratio. Chromosomal measurements are usually done on highly enlarged photographic prints or Camera Lucida drawings (Yunis, 1965).

Turpin and Lejeune (1969) reported a conspectus of human, mitotic chromosomes in groups.

Group A (Chromosomes 1-3) - large chromosomes with approximately median centromeres. The three chromosomes are readily distinguished from each other by their size and centromere position.

Group B (Chromosomes 4-5) - large chromosomes with distal centromeres. These two chromosomes are difficult to distinguish, but chromosome 4 is slightly longer than chromosome 5.

Group C (Chromosomes 6-12) - medium-sized chromosomes with submedian centromeres. The X-chromosome resembles the longer chromosomes in the group, especially chromosome 6, from which it is difficult to distinguish. This group is the one which presents greatest difficulty in the identification of individual chromosomes.

Group D (Chromosomes 13-15) - medium-sized chromosomes with nearly terminal or acrocentric chromosomes. Chromosome 13 has prominent satellites on the short arms and chromosome 14 has small satellites on the short arms. No satellites have been detected on chromosome 15.

Group E (Chromosomes 16-18) - rather short chromosomes with approximately median centromeres (in chromosome 16) or submedian ones.

Group F (Chromosomes 19-20) - short chromosomes with approximately median centromeres.

Group G (Chromosomes 21-22) - very short acrocentric chromosomes. Chromosome 21 has satellites on its short arms. The Y-chromosome is similar to these chromosomes.

Yunis (1965) has described each chromosomal group in detail with relation to many different methods of identification of chromosomes. German in 1970 referred to the short arm of a chromosome with the letter p, the long arm of a chromosome with q, and a minus sign (-) to indicate an abnormal shortness of the arm.

Bender and Kastenbaum (1969) noted that the late-labeling autoradiographic technique, which is often spoken of as a means of identifying individual pairs of autosomes within the difficult groups, suffers from many problems in pairing on the basis of arm lengths. Pairing of autosomes by this means of labeling patterns can be shown to be a valid means of identification. Yunis (1965) states that a number of investigators have applied the autoradiographic method to human chromosomes with attention focused on the asynchronous deoxyribonucleic acid (DNA) replication pattern of the X-chromosomes in females and in human sex anomalies having increased numbers of X-chromosomes. The studies showed that, in cells having two or more X-chromosomes, only one X completes synthesis of DNA at a rate and time comparable with the general patterns of the autosomes; the

additional X chromosomes show a much higher rate of DNA synthesis toward the end of the DNA synthetic period, and are the last chromosomes in the complement to complete DNA replication (Yunis, 1965). The late-labeling autoradiographic technique is therefore a useful method in identifying the X-chromosomes. Examination of the labeling pattern the chromosomes produce in autoradiograph when they reach metaphase makes it possible to identify various members of the complement by their characteristic patterns (German, 1970).

LITERATURE REVIEW

Detectable abnormalities in human chromosomes, both numerical aberrations and structural changes, have attracted increasing interest in the fields of medicine and biology because of their importance as causes of abortion, birth defects, and mental retardation. Approximately 3 out of every 100 human pregnancies are attended by visible anomalies among the child's chromosomes. The changes are usually accompanied by clinical symptoms including severe malformations and miscarriage, Mongoloid idiocy, and slight or extreme effects on the sexual characteristics of the mature offspring (DuPraw, 1970). Polani (1963) estimated the overall incidence of chromosomal abnormalities responsible for birth defects in the general population to be of the order of 0.5 percent.

The forms of chromosomal abnormalities consist of numerical and structural abnormalities. Bartalos and Baramki (1967) describe numerical anomalies as a variation in the total number of chromosomes either as an exact multiple of the haploid number (euploidy) or by deviation from this multiple (aneuploidy). The most common situation of the former type is characterized by the presence of more than two haploid sets of chromosomes. This polyploidy may be due to various mechanisms leading to chromosomal duplication without subsequent cell division. Aneuploidy

includes both hypoploid types (monosomy) where one or several chromosomes have been lost (2n-a) and hyperploid types (trisomy) where the nucleus has acquired extra chromosomal material (2n+a). Non-disjunction and anaphase lagging are the chief mechanisms leading to aneuploidy. In both cases, genetically different cell lines are produced by these abnormal mechanisms. One cell is chromosomally deficient, and the other contains either a normal or a hyperploid chromosome number.

Turpin and Lejeune (1969) consider structural abnormalities to be of two general types: those which entail a loss or gain in chromosome material and those which correspond to a rearrangement in structure without appreciable change in quantity. Deletions, duplications, and translocations correspond to these structural rearrangements.

Most structural abnormalities of the chromosomes are considered consequences of chromatid and chromosome breaks. A strong tendency to heal by becoming attached to other similarly broken ends is characteristic of the breaks in chromatids and chromosomes. A chromosomal abnormality is created if the broken end becomes attached at an abnormal site of its homologous pair, or is transferred to a heterologous chromosome (Saxen and Rapola, 1969). Mittwoch (1967) describes isochromosomes as chromosomes in which two homologous arms are joined at the centromere and as compared with normal chromosomes, represent a duplication

of one arm and a deficiency of the other arm. If two breaks occur on opposite sides of the centromere, the ends of the piece containing the centromere may fuse forming a ring chromosome losing two acentric fragments and is therefore a deleted chromosome. A translocation results if a break occurs in two different chromosomes and the broken ends rejoin reciprocally forming a reciprocal translocation between the chromosomes. This is the most common type of translocation.

Of the 22 possible autosomal trisomies, only trisomy 13(D_1), 18, and 21(G_1) are known to be compatible with life (Yunis, 1965). Trisomy, of any except a sex chromosome and the three autosomes listed, produces a lethal genetic imbalance; however, trisomy of other autosomes have been detected among human embryos spontaneously aborted during the first few weeks of gestation. No viable autosomal monosomy has yet been observed (German, 1970).

Of these autosomal trisomies, the G_1 trisomy (known as Down's syndrome, trisomy 21 syndrome, or mongolism) is by far the most common. Lejeune et al. (1959) reported that mongolism is the developmental consequence of a trisomy of the largest chromosomes of the G complement, chromosome No. 21. German (1970) states that trisomy 21 is important not only because of the severity of the retardation accompanying it but because of its frequency, approximately 1 in 650 liveborn children. It has been shown that with increased maternal age, there is a greater rate of

non-disjunction of this chromosome. Recent estimates of Turpin and Lejeune (1969), Carter et al. (1961) and Penrose (1961) are in full agreement with others that before the age of 30, the risk is of the order of 1 in 2,000; after the age 35, it increases to 4 in 1,000; and rises to a value of 2 per cent after the age of 45.

The facial characteristics of persons with trisomy 21 permit very early clinical diagnosis usually at birth. These include a round, full face, turning downward of the upper eyelids, and malformation of the ears (Winchester, 1966). Yunis (1965) stated the most frequent characteristics besides mental retardation, include: short stature, brachycephaly, flat occiput, epicanthic folds, oblique palpebral fissures, hypertelorism, strabismus, mystagmus, cataract, furrowed tongue, characteristic dermatoglyphic patterns, irregular and abnormal teeth, narrow, high palate, a short nose, abnormal ear lobes, short neck, short broad hands, short incurved fifth finger, clinodactyly, a gap between great and second toe, protuberant abdomen, and hypotonicity. Longevity is reduced in patients with the syndrome because of frequent association of congenital heart lesions, especially septal and atrioventricular canal defects, duodenal atresia, frequent pulmonary infections, and leukemia. The karyologic basis of the disorder shows that more than 95 percent of the mongoloids are true trisomies of one of the group G chromosomes (21-22). In some cases, the extra G material is found translocated to

other chromosomes, usually to those of the D group (Saxen and Rapola, 1969).

An autosomal disorder associated with an extra chromosome in the group (13-15) was reported in 1960 by Patau et al. This trisomy for a medium sized acrocentric of the 13-15 group was rapidly confirmed by the authors and then by others (Turpin and Lejeune, 1969). As in mongolism, trisomy D_1 syndrome is generally produced by non-disjunction. Most of the known cases of D_1 trisomy have occurred sporadically in the population. Ferguson-Smith (1962) examined 18 patients with D_1 trisomies and found 61 percent to be female, but more cases are required before the D_1 trisomy sex ratio can be considered significant. The incidence of D_1 trisomy has been reported as 0.45 per 1,000 newborns (Yunis, 1965). Turpin and Lejeune (1969) describe these children as underdeveloped with multiple malformation, profound mental retardation, small skull, mainly through aplasia of the frontal and parietal regions, small eyes with true microphthalmia ranging from anophthalmia to iridal colobomata, sloping forehead, low set malformed ears, cleft palate and lips, polydactyly and/or syndactyly, heart defects, usually interventricular septal defects, and capillary hemangiomata. Yunis (1965) includes other frequent findings as deafness, fused or webbed fingers and toes, seizures, hyperconvex narrow fingernails, flexed and overlapping fingers, rockerbottom feet, abnormal calcification of skull, accessory spleens,

retroflexible thumbs, urinary anomalies, large gallbladder, incomplete rotation of the colon, umbilical hernia, cryptoorchidism and abnormal scrotum in males, and partially bicornate uterus in females.

Edwards et al. (1960) and Patau et al. (1960) described simultaneously, a new syndrome associated with the presence of an extra group E chromosome (16-18) considered to be chromosome 18. Affected individuals have very limited life spans usually dying in early infancy, but occasionally may survive into early childhood. There is approximately a 3:1 female-to-male sex ratio; this difference may, in part, reflect increased intrauterine lethality in males. The occurrence of primary non-disjunction as in mongolism is considered to be the usual cause of this syndrome (Hecht et al. 1963).

Turpin and Lejeune (1969) suggested the frequency of trisomy 18 to be one to two per 1,000 births. The age of the mother, as in mongolism, distinctly influences the appearance of the disease. Hecht et al. (1963) reported the mean maternal age in trisomy 18 to be 34.3 years as compared to 26.4 years for the general population. The most constant features of this syndrome are the failure to thrive, neurological retardation, malformed low set ears, small mandible, flexion deformities and overlapping of fingers, and hypertonicity. Other frequent findings include: umbilical and/or inquinal hernia, Meckel's diverticulum, heart defects, especially interventricular septal

effects, and patent ductus arteriosus, dorsiflexed short big toe, limited hip abduction, foot deformities, short sternum, small pelvis, urinary tract anomalies, eventration of the diaphram, heterotopic pancreatic tissue, prominent occiput, and retarded osseous development (Rosenfield et al., 1962 and Yunis, 1965). Although most cases of trisomy 18 are typical trisomies, some cases of trisomies by translocation are known.

Uchida et al. (1964) state that the cytological explanation for those conditions which exhibit only some of the anomalies that make up a syndrome, is usually mosaicism, or translocation. The term "partial trisomy" has been suggested by Patau et al. (1961) for those cases in which only a segment of a chromosome is present in triplicate as the result of a translocation. Deletions of minor fragments have frequently been found in children with serious congenital defects.

Lejeune et al. in 1963, observed a new clinical entity attributed to an isolated deletion. The chromosomal aberration is characterized by loss of about half the length of the short arm of one of the members of chromosome 5 (Turpin and Lejeune, 1969). Very few of these cases have been described and McGavin et al. (1967) state only about 50 cases (16 males and 34 females) have been reported. German (1970) refers to this abnormality as the 5p- syndrome. The most common abnormal feature of these patients is their particular cry which greatly resembles a cat mewing;

therefore, the condition is also called the "cri-du-chat" syndrome. Low birth weight, slow growth, pyschomotor retardation, a small round face, microcephaly, widely spaced eyes, and certain other defects seem to be relatively common in the syndrome.

The 4p-, 18p-, and 18q- syndromes are three other deletion syndromes which have subsequently been recognized and each having clinical features often distinctive enough to permit at least a strong suspicion of the correct diagnosis even before the chromosomal analysis. Characteristic features of the 4p- syndrome include an unusually small patient with severe psycho-motor retardation, convulsions, a wide flat nasal bridge, a prominent forehead, cleft palate, and congenital heart disease (German, 1970). Grouchy et al. (1963) described a severely mentally retarded boy with a karyotype of 46 chromosomes revealing the absence of a chromosome 18 replaced by a telocentric, the arm of which was identical in size to that of the long arm of 18. Characteristic features of the 18p- syndrome include: hypertelorism with incomplete convergence in the right eye, low set ears, deformed hands with high setting of the thumb and curving of the fifth finger, and deformed feet with syndactyly of the third and fourth toes. The most plausible explanation for the anomaly is loss of the short arm of 18 just above the centromere. The 18q- syndrome is characterized by a deletion of the long arms of chromosome 18. This syndrome was discovered by Grouchy et al.

in 1964 and has since then been documented in more than 24 cases. Small stature, mental deficiency with hypotonia, conductive deafness, microcephaly, nystagmus, and abnormal genitalia are characteristic abnormalities of the 18q- syndrome. Another deletion syndrome, 21q-, has been reported in three cases which appears to be a distinct entity but requires further definition before a clear pattern of malformation can be set forth (Lejeune et al., 1964).

Sex chromosomal abnormalities, like autosomal chromosome aberrations, can be either numerical or structural. The study of the sex chromatin pattern as a companion technique to chromosome analysis allows one to determine the number of X chromosomes in the complement, according to the rule that the maximum number of sex chromatin masses is one less than the number of X chromosomes. This is particularly helpful in certain chromosome abnormalities because the X chromosome is almost identical with autosomes 6 and 7. The neutrophile method of nuclear sex detection is based on the occurrence of occasional neutrophile leucocytes that have a drumstick-shaped appendage in normal females, but not in normal males. This accessory nuclear lobule is thought to contain sex chromatin (Yunis, 1965).

Nearly two-thirds of the known chromosomal anomalies in liveborn children consist of abnormalities, most of which are numerical aberrations, of the sex chromosomes (Saxen and

Rapola, 1969). Human sex chromosome aberrations give rise to two major clinical disorders which are best known eponymously as Klinefelter's and Turner's syndromes with the Klinefelter syndrome more common (Ferguson-Smith, 1966).

In 1942, Klinefelter et al. described a clinical condition in male patients who suffered from increased development of the breasts, lack of spermatogenesis, and increased excretion of follicle stimulating hormone. Another name sometimes used to describe this condition is "gonadal dysgenesis" (Mittwoch, 1967). The individuals develop as males but have small testes, are sterile, and usually have a subnormal IQ (DuPraw, 1970). In 1959 Jacobs and Strong found that a patient with Klinefelter's syndrome had 47 chromosomes, interpreted as including an extra X chromosome in addition to the normal XY complement. This provided the first concrete evidence that abnormalities of sexual development may be caused by an abnormal sex chromosome constitution. Other chromosome combinations can give rise to essentially the same clinical condition, but the XXY chromosome constitution has proved to be the most common one in Klinefelter's syndrome. Some combinations which have been described include: XXXY sex chromosome constitution (48 chromosomes in all), 49 chromosomes and XXXXY sex chromosomes. Some patients with the syndrome have abnormalities of the sex chromosome constitution which are not the same in all the cells of the body, making human chromosomal mosaics. These chromosomal mosaics include

XY/XXXY sex chromosomes, XX/XXY, XY/XXY, XXXY/XXXXY, and others, including some triple mosaics (Mittwoch, 1967).

The XX/XXX mosaic has been described by Grouchy et al. (1961) and Jacobs et al. (1961). The female who furnished this type of mosaic was not distinguishable at first sight from a normal female but had only menstruated once at the age of 21 years (Grouchy et al., 1961). Another young woman of normal body form with primary amenorrhea at the age of 19 years who had a XX/XXX chromosomal constitution was reported by Jacobs et al. (1961).

Klinefelter's syndrome may also be subdivided into chromatin-negative and chromatin-positive varieties. The chromatin-negative patients have normal karyotypes and show two types of testicular abnormality; in one there is post-pubertal testicular atrophy with evidence of interstitial cell failure and in the other there is a primary deficiency of germ cells. The only etiological factor of note is an increased maternal age. Chromatin-positive cases are caused by abnormal sex chromosome complements that lead to the differentiation of testes that are defective in germ cells and in interstitial cell function. A sex-limited autosomal dominant, or X-linked recessive, type of inheritance of a mutant gene may occur in some cases involving chromatin-positive patients. The major implication of sex chromatin surveys seems to be that patients with chromatin-positive Klinefelter's syndrome are inherently more prone to a wide variety of physical and

mental disorders because of their abnormal genotype. The incidence of the syndrome (chromatin-negative and chromatin-positive) increases with maternal age with the effect of age being so great that if all mothers could complete their families by the age of 35 years, the present incidence of Klinefelter's syndrome could be reduced by 40 per cent (Ferguson-Smith, 1966). The origin of one or more additional sex chromosomes in Klinefelter's syndrome is presumed to be due to non-disjunction (Mittwoch, 1967).

Another major clinical disorder related to human sex chromosome aberrations is Turner's syndrome which is one of the numerous names given to the clinical entity in women. Many authors prefer "gonadal dysgenesis" and "ovarian dysgenesis" (Mittwoch, 1967). The most common anomalies include: webbed neck, broad shield-like chest and rudimentary nipples, lymphedema of the extremities, coarctation of the aorta, various skeletal deformities, microthelia, cutaneous, ocular, otic and renal abnormalities, and mental retardation but not so common nor so severe as in Klinefelter's syndrome (Moore, 1966). In 1954, it was shown by Polani *et al.* and by Wilkins *et al.* that the majority of patients with Turner's syndrome lacked sex chromatin, and then in 1959, Ford *et al.* and Fraccaro *et al.* demonstrated that the sex chromosome constitution of these patients consisted of a single X chromosome, giving a total chromosome number of 45. There are many combinations of sex chromosomes which can give rise to essentially

the same phenotype, although the XO chromosome constitution is the most common in Turner's syndrome. The possible variations include the presence of a structurally abnormal sex chromosome, mosaicism, and a combination of the two (Mittwoch, 1967). Turpin and Lejeune (1969) reported that drumsticks are often absent in the cells. Eighty percent of the patients with Turner's syndrome have chromatin-negative nuclei despite their essentially female anatomy (Moore, 1966).

True hermaphrodites have gonads containing both ovarian and testicular tissue with the distribution of these tissues varying in different patients. Sex chromatin has been found in the majority of such patients and chromosome studies have shown that most hermaphrodite patients have XX-sex chromosomes. A minority of hermaphrodite patients have an XY-sex chromosome constitution. All human hermaphrodite patients are sterile (Mittwoch, 1967).

The study of human chromosomes is contributing greatly to the diagnosis of specific clinical entities in patients with abnormal sex development, sterility, mental deficiency, and certain congenital anomalies. Chromosome studies in relatives of individuals with autosomal chromosome abnormalities are also useful to the genetic counselor in providing guidance for parents of affected children (Yunis, 1965).

MATERIALS AND METHODS

Preparation and Incubation of Cells

Preparation of peripheral blood leukocytes for chromosomal analysis was accomplished with the use of the TC-Chromosome Culture Kit obtained from Difco Laboratories. Venous blood from a patient who had abstained from eating for at least 3 hours was asptically withdrawn and transferred to a Blood Separation Vial containing aqueous heparin (1000 units/ml). The volume of blood withdrawn varied from 2-10 ml depending upon the patient involved. The blood and heparin were mixed by inversion and the vials incubated at 37°C for 1-3 hours or until the Plasma-Leukocyte suspension separated.

Each bottle of Chromosome Medium was reconstituted with a vial of warm (37°C) Chromosome Reconstituting Fluid. The Chromosome Medium consists of Hanks' 199 base plus 20% fetal bovine serum adjusted to pH 7.2 to 7.5. Penicillin (200 units/ml), dihydrostreptomycin sulphate (200 ug/ml) and phytohemaglutinin-P (0.1 mg) are incorporated in the medium. Each bottle of rehydrated Chromosome Medium was inoculated with 1.5-2.5 ml of Plasma-Leukocyte suspension from the Blood Separation Vial at the end of the incubation period. The transfer was performed with a sterile syringe fitted with an 18 gauge needle.

Incubation of Culture

The inoculated Chromosome Medium bottle was incubated in an upright position at 37°C for 3 days or approximately 72 hours at which time the mitotic index is at a maximum. Maintaining the proper pH range of the culture at all times was very important; therefore, close observation of the indicator color was made at various intervals during incubation.

At the end of the incubation period, arresting fluid containing 2 ml colcemid (4.0 ug/ml) in Hanks' balanced salt solution (BSS) was added to the culture to halt mitosis. One hundred units per ml of penicillin are incorporated in the fluid. The arresting solution terminates mitosis at metaphase. This mixture was then incubated an additional 3-6 hours at 37°C after which the cells were suspended by means of a sterile syringe with an 18 gauge needle.

Harvesting and Fixation of Cells

The cell suspension was transferred to a 12 ml graduated conical centrifuge tube and centrifuged for 12 minutes at 800 rpm in a clinical centrifuge. The supernatant was decanted and 5-6 ml of warm (37°C) Hanks' BSS was then added; the cells were resuspended using a Pasteur pipette with bulb and the suspension centrifuged at 800 rpm for 5 minutes. All but 0.5 ml of the supernatant was aspirated off with the pipette. The packed cells were

resuspended in this volume and 1.5 ml of warm (37°C) distilled water were added slowly while shaking to produce a hypotonic solution to aid in separation of chromosomes. The cell suspension was then incubated at 37°C for 10 minutes. The Leukocytes were centrifuged at 600 rpm for 5 minutes and the supernatant aspirated off.

A 3-4 ml volume of freshly prepared fixative consisting of 1 part glacial acetic acid and 3 parts methanol was added slowly so as not to disturb the button of cells. The cells were left to soak in the fixative for 30 minutes and then resuspended with the pipette. The suspension was centrifuged at 600 rpm for 5 minutes and the supernatant discarded. The cells, resuspended in 3-4 ml of fresh fixative, were left to soak for 5 minutes and then centrifuged at 600 rpm for 5 minutes. The supernatant was aspirated off and 0.25-0.5 ml of fresh fixative was added to the button of cells. The cells were resuspended using a pipette to get a hazy suspension.

Preparation of Slides and Microscopy

The microscope slides were cleaned in acid, rinsed thoroughly with distilled water, and chilled in a beaker of distilled water in the refrigerator. The excess water was shaken off each chilled microscope slide and 2-3 drops of the cell suspension were added using a pipette. The slide was immediately tipped a few times to spread the suspension of cells and then the excess fixative was ignited

by bringing it momentarily in contact with a flame. The slide was waved vigorously to hasten drying, this was accomplished as rapidly as possible without letting the slide get hot.

The slides were placed in a small staining dish and covered with Giemsa Stain diluted 1:10 in distilled water. The slides were allowed to stain for approximately 20 minutes or until adequately stained, then rinsed in distilled water and left to air dry.

The slides were scanned with a binocular Unitron Microscope with a 40x objective and a 10x eyepiece. When a suitable chromosome spread was found, the slide was transferred to a Spencer Microscope to be photographed.

Photography

Photographs were made on a monocular Spencer Microscope with an oil immersion objective at a magnification of about 950x using the 95x objective (NA=1.25) and a 10x ocular. The microscope was illuminated by an adjustable intensity lamp equipped with a tungsten ribbon-filament bulb. Best results were obtained using orange, yellow (k-2), and blue filters.

An Exakta camera with either Panatomic-X or High Contrast Copy films were used for the photographs. The Panatomic-X film was developed in Kodak Microdol-X and the High Contrast film in Kodak D-19. Prints were made on Kodabromide F-5 single weight photographic paper.

Analysis

The enlarged prints of the chromosomes from the individual cases were used in making the karyograms for analysis. The chromosomes, cut individually from the enlargement, were positioned according to group and then each was paired with its homologue with respect to size and centromere position. The chromosome pairs were then rearranged in numerical order within each group. A reduced print of the entire chromosome spread was also made to be included in the karyogram.

RESULTS

Karyotypic analyses of seven patients were made to detect chromosomal anomalies and identify the type of abnormalities involved. The analyses were made from patients thought to possess a chromosomal abnormality. Of the seven patients, four were females and three were males.

The first karyotype was made of a 6 day old caucasian female. The infant weighed 5 lb. 12 oz. at birth and was born to a 27 year old woman terminating a 38 week uncomplicated pregnancy. A previous pregnancy had ended in a spontaneous abortion after 14 weeks with no known abnormalities in the fetus. The most outstanding clinical observation of the 6 day old infant was gastroschisis to the right of a normally positioned umbilical cord. The eviscerated mass contained the entire mesenteric small intestine and colon and was covered by a meconium-stained, thickened, glutinous peel. The small intestine appeared to be foreshortened and the colon was greatly distended with meconium. Incomplete rotation of the intestine with a right-sided duodenum was also noted with the third portion of the duodenum obstructed by bands of Ladd. A ventricular septal defect was detected and after death of the infant by aspiration, the arteries and veins to and from the heart were found to be in reverse position. A karyogram was constructed and found to be normal as shown in Figure 1.

The second case involved a 17 year old caucasian female with certain characteristic masculine features including body build, facial features, and hair growth. The primary observed characteristics were broad shoulders with the constitutional build of a male and a rather broad neck. Hair growth was more masculine than feminine with the hair growing further down the neck, upper and coarse hair on the arms, and also low hairgrowth on the forehead with some hirsutism of the face including over the lip.

The patient had been having menstrual irregularities with unpredictable menstrual periods every since menarche at age 12 and gross abnormalities of menstrual flow. Upon medical examination, she was found to have normal external and internal genitalia although the cervix was slightly to the right of the midline. There were no associated endocrine abnormalities. Construction of the karyogram (Figure 2) showed a normal chromosomal constitution.

The third karyotype involved a 19 year old male mongoloid. The mother was 38 years old at the time of childbirth. Observed characteristics of the patient included: mental retardation, short stature, a round, full face, brachycephaly, flat occiput, epicanthic folds, irregular and abnormal teeth, short nose, abnormal ear lobes, short neck, short broad hands with incurved fifth finger, protuberant abdomen, and hypotonicity. Each cell counted had 47 chromosomes. The karyogram (Figure 3) revealed the cells to be typical trisomies of chromosome 21.

A karyotypic analysis was made of an 8 month old female born to a 27 year old mother. The pregnancy and delivery had been normal. Observations at birth included: small mandible, bilateral dislocated hips, bilateral simian lines, hyposensitive heart murmur, low set ears, high arched palate, antimongoloid slant to eyes, and a most pronounced cry resembling the mewing of a cat. However, all characteristics at birth were not in full agreement for classification as a "cri du chat" syndrome. Karyotypic analysis (Figure 4) revealed the deletion of a portion of the short arm of chromosome 5.

The fifth karyotype (Figure 5) was constructed of a 4 month old male and the infant was found to exhibit Down's Syndrome as predicted from clinical observations. The mother was 21 years old and the father 23 years of age when the infant was born. The child possessed the typical trisomy 21 characteristics as previously stated but due to the age of the parents and because the infant was the first born, karyograms were also made of both parents. As shown in Figure 6, the father of the infant was normal.

As can be seen from the photographs (Figures 7-10), much difficulty was encountered in the construction of the mother's karyograms. Some cells were determined to have a normal karyotype (Figures 7-8), but in other cells (Figures 9-10) two of the chromosomes could not be satisfactorily paired.

DISCUSSION

Analyses of human chromosomes have been a challenge to researchers for many years. The cytological techniques involved are tedious and complex. The difficulties encountered in this study involved certain aspects of culturing, harvesting, staining, and photographing. A few cultures were completely lost due to acidity. The caps on the culture bottles were not loosened to allow the carbon dioxide produced to escape, and therefore, resulted in an acid condition which reduced the mitotic index. In some cultures no mitotic figures were observed on any of the slides. Other cultures became acidic even though caps were loosened, probably resulting from too many cells in the culture.

The main difficulty encountered in harvesting the cells was clumping. When the cells were suspended in the medium, large aggregates of tissue were observed. Before continuing the procedure, these clumps were removed and only those cells which were easily suspended were harvested. Suspension was accomplished with the use of a syringe and 18 gauge needle.

Staining of the chromosomes provided other problems. A dilution of Geimsa and distilled water had to be found that would not fade after a limited period. Due to the microscope used for photography, the chromosomes had to be

slightly over stained for good contrast. A dilution of 1:10 in distilled water and a staining time of approximately 20 minutes produced the best results. The most critical step in photography was the type of film. After many unsuccessful experiences with Panatomic-X, High Contrast Copy Film was found to give the best contrast.

From the karyotypic analysis made of the female infant with gastroschisis to the right of a normally positioned umbilical cord, it was concluded that the infant did not possess a chromosomal abnormality which could have caused the abnormal phenotype. Phenotypic abnormalities may therefore occur at birth and not necessarily be caused by anomalies of the chromosomes.

Many clinical entities associated with congenital malformations have been studied for various reasons and found to have normal chromosomal patterns. The infant included in this study with gastroschisis had a normal chromosomal constitution with definite congenital malformations. A review of malformation syndromes in which the karyotypes were apparently normal was attempted by Harnden (1961).

The most striking among the large number of syndromes studied by Harnden (1961) include DeLange's syndrome (typus degenerativus amstelodamensis) which does not involve detectable chromosomal anomalies according to Hienz (1963) and Laurence and Ishmael (1963) but distinct congenital malformations have been observed. About 120

cases have been reported with the outstanding characteristics being mental retardation, sluggish physical activity, synophrys of eyebrows, thin downturning of the upper lip, and micromelia (Smith, 1970). Another syndrome with a normal karyotype is the Laurence-Moon-Biedl syndrome (Böök et al., 1961) which has principal abnormalities of mental deficiency, obesity, polydactyly, and retinal pigmentation. Smith (1970) stated that more than 300 cases of this syndrome have been reported, with no associated chromosomal anomalies.

The other case in this study of an unusual phenotype with a normal karyotype was that of the 17 year old female with certain masculine characteristics and secondary amenorrhea. Karyotypic analysis indicating XX/XXX mosaics have been reported by Grouchy et al. (1961) and Jacobs et al. (1961) concerning females with normal body builds and menstrual irregularities which gave reason for suspecting the patient to be a possible XX/XXX mosaic. The description of the patient given by her physician included many of the characteristics associated with Turner's syndrome as stated by Turpin and Lejeune (1969). However, consideration of two major constant features of Turner's syndrome, small stature and primary amenorrhea, ruled out the possibility that the patient possessed this particular syndrome. The definite abnormal phenotype of the patient caused much interest, therefore, an analysis was made for determination. The karyotype constructed and chromosome counts made

indicated the female to have a normal chromosome complement. The explanation for her unusual masculine characteristics has not been determined.

The 19 year old trisomy 21 male is a rather common occurrence considering the mother's age of 38 years at the time of birth of the son. The woman also has a 22 year old normal daughter. Turpin and Lejeune (1969) and others have established the fact that there is an increase in the frequency and risk of a child being born with Down's syndrome to a mother over the age of 30. German (1970) states that with increased maternal age, there is a greater rate of non-disjunction of chromosome 21. Considering the karyogram constructed of the patient and the increased maternal age at the patient's birth, this trisomy 21 male most certainly must be a resultant of primary non-disjunction of parental chromosomes. The importance of trisomy 21 should definitely be stressed because of its frequency.

The 8 month old female with the "cri-du-chat" syndrome is a rather unusual case in that very few of these cases have been reported. McGavin et al. (1967) state only 50 cases have been described. The first was observed by Lejeune et al. in 1963 finding a new clinical entity attributed to an isolated deletion of one of the B group chromosomes. In 1964, German et al. demonstrated by autoradiographic studies that this was chromosome 5. The karyotypic analysis (Figure 4) of the child shows the partial deletion of the short arms of chromosome 5. The

patient possessed a number of the characteristics of the syndrome, especially the pronounced cry resembling the mewing of a cat. Pyschomotor retardation has also been noticed in the child.

The karyotypic analysis (Figure 5) made of the 4 month old male provided cytological evidence of trisomy 21 as suspected. The infant exhibited the facial characteristics of a round, full face and downward slant of upper eyelids at birth which permitted early clinical diagnosis of Down's syndrome. The analysis was made to provide unequivocal evidence of trisomy 21.

The most outstanding feature in regard to this latter case is the age of the parents. Since the syndrome occurs more frequently in older parents, the age of the mother (21 years) and the father (23 years) caused much concern. Yunis (1965) states that only 20 percent of the trisomy 21 cases are born to younger parents. The term "younger parents" usually refers to parents under 30 years, in this case the mother may be described as "extremely young." Primary non-disjunction, translocation, or mosaicism are the major causes of the syndrome. Primary non-disjunction is sporadic in the population with no predictable pattern except an increase with maternal age. Mongolism due to a translocation would be recognized if one parent had only 45 chromosomes, since reports of group G translocated to other chromosomes involves a reduced chromosome number. The parent carrying the translocation

is usually the mother and she need not show an abnormal phenotype.

Mosaicism involves two different cell populations, one trisomic for chromosome 21 and the other normal (Yunis, 1965). Here again there may not be an associated phenotypic abnormality.

Karyotypic analyses of the parents were therefore made to enable an explanation for the abnormal child and determine the risk of recurrence. The karyogram (Figure 6) of the father was found to be normal.

Much difficulty was encountered in the construction of the mother's karyograms. Sixteen karyograms were made from different metaphase spreads with 46 chromosomes in each. Normal karyograms (Figures 7-8) were constructed and the chromosomes easily paired. There were others which had two chromosomes which could not be paired satisfactorily with any of the other chromosomes. After much consideration, the unmatched chromosomes were placed together and identified as the X chromosome pair on the karyograms (Figures 9-10). It is believed that the larger chromosome of the unmatched pair is the sex chromosome. The smaller chromosome could possibly be a deleted X chromosome (Xq-) or one of the shortest of the C group. It is also possible that the X chromosome as shown in Figure 9 and 10 could match with one member from chromosome 6 and give a normal sex chromosome complement. In either event, one of the largest chromosomes in the C group, either chromosome 6 or

the X chromosome will be without a homologue. Experimentation with mice provides reason to believe the human X chromosome could possibly exist without a homologue. Russell and Saylors (1960) state that XO mice are phenotypically normal and fertile females. It is therefore possible for the human female lacking a portion of the X chromosome to be phenotypically normal and fertile.

It may also be noted that some of the other pairs do not match as well as would be expected. This can be seen in the chromosome 3 pair in Figure 9 and chromosomes 1 and 4 in Figure 10. However, these do not represent a major problem, since the variation in these may be explained in terms of varying degrees of condensation or elongation of the arms. This explanation does not resolve the problem of the unmatched X pair.

Yunis (1965) has described a case which may be similar to the one reported here. The report involved phenotypically normal parents, ages 24 and 23. Three successive children with trisomy 21 were born. Karyotypes of both the mother and father were apparently normal. However, it is suspected that one of the parents is a mosaic. It is noted with great interest that the ages of the parents reported by Yunis coincide with the ages of the mother and father in these findings. Another case of familiar tendency toward non-disjunction has been described by Miller et al. (1961) but no explanation was offered for the observed results.

If the female in this study is indeed a mosaic, then this abnormal karyotype is different from any reported in the literature. Since the abnormal constitution involves group C, it is impossible to resolve the discrepancy with any measure of certainty. Consultation with others involved with human cytogenetics also yields ambiguities. The explanation of the mother's unusual karyotype has not been determined.

SUMMARY

Karyotypic analyses were made of four females and three males. Of the patients examined, two were phenotypically normal and five exhibited abnormal phenotypes. From chromosomal analyses, three normal and three abnormal karyotypes were constructed and one patient is thought a possible mosaic.

The first two patients were found to be of normal karyotype even though both females were phenotypically abnormal.

A male exhibiting Down's syndrome was examined and consistently gave a chromosomal count of 47. The karyotype revealed that the extra chromosome was of the G group.

A phenotypically abnormal female was clinically observed as a "cri-du-chat" syndrome. Karyotypes constructed showed a deletion of the short arm of one of the members identified as chromosome 5, thereby confirming the clinical diagnosis.

A male infant with classical mongoloid features was observed to be a typical trisomy 21. This case created much interest because of the young age of the parents. Karyograms of the father (23 years) were normal. However, analysis of the mother (21 years) indicated the possibility that there were 2 different cell populations. One cell type was concluded normal; the other is thought to have an

abnormal chromosome constitution. The two abnormal chromosomes cannot be paired satisfactorily with any of the others. It is believed that the largest of the two is the X chromosome. The smaller member resembles the shortest chromosomes of the C group.

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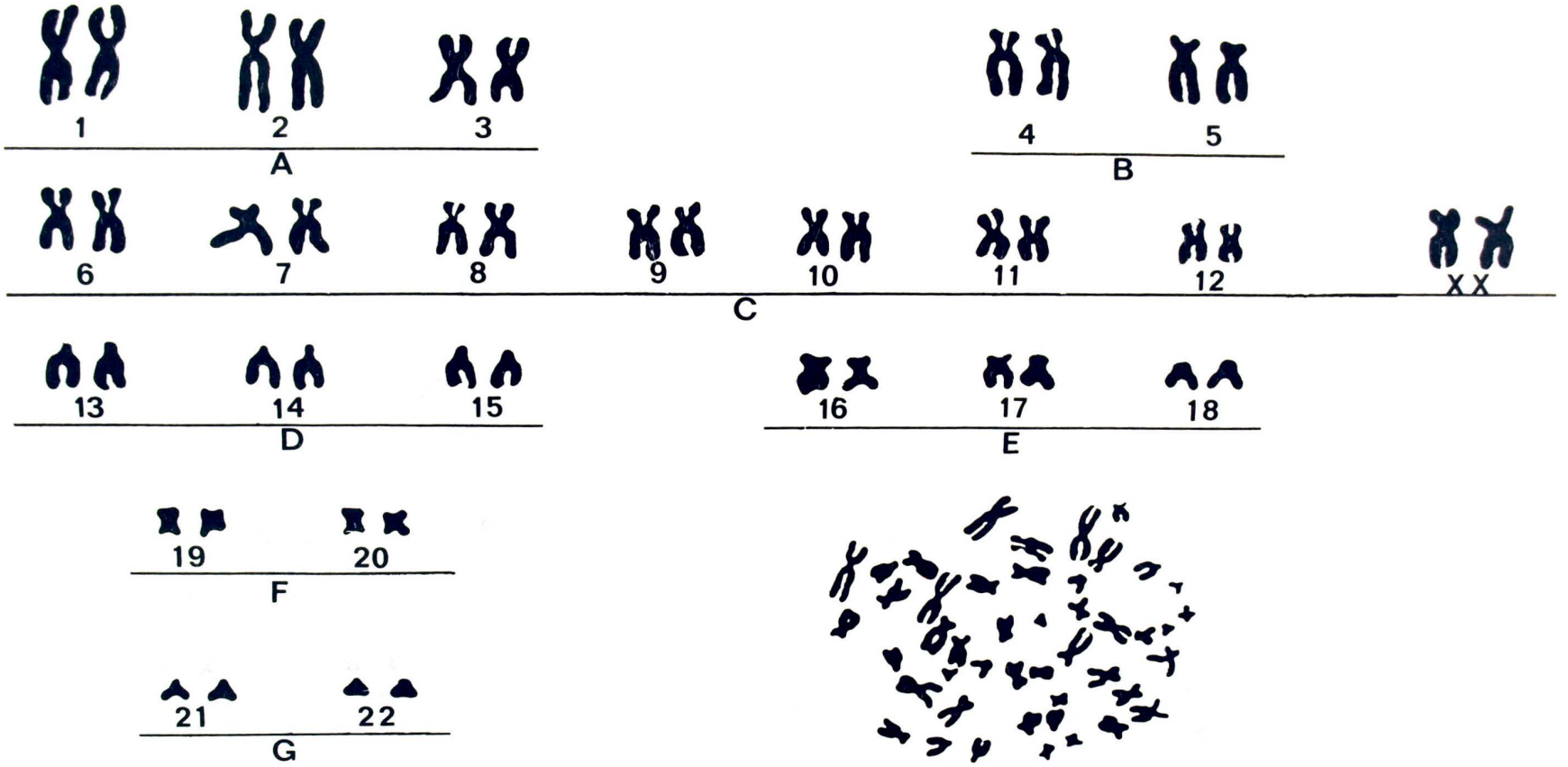

Figure 1. Karyogram with Normal Chromosome Complement of Infant Female with Gastroschisis.

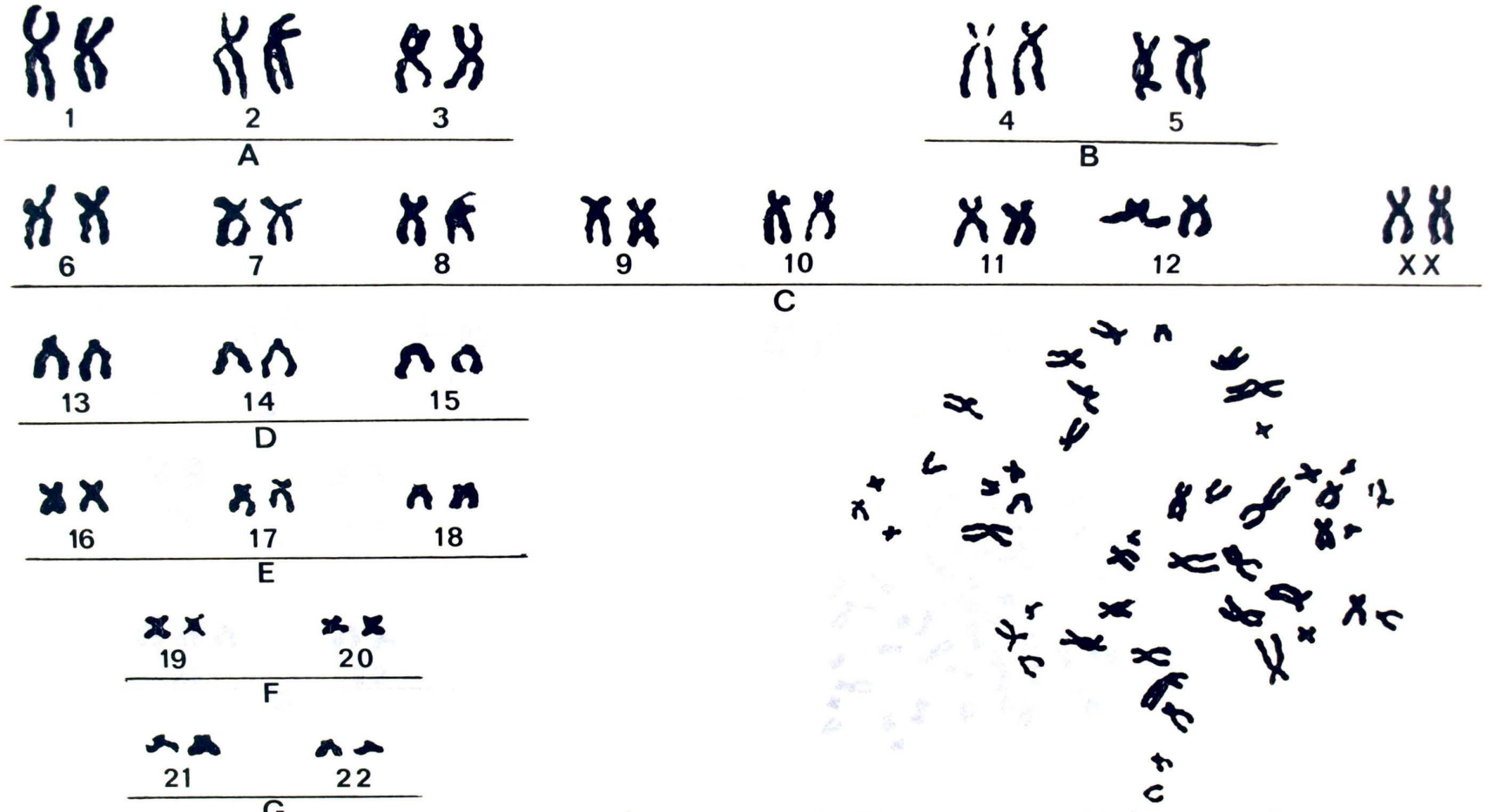

Figure 2. Normal Karyogram of a 17 Year Old Female with Certain Masculine Features.

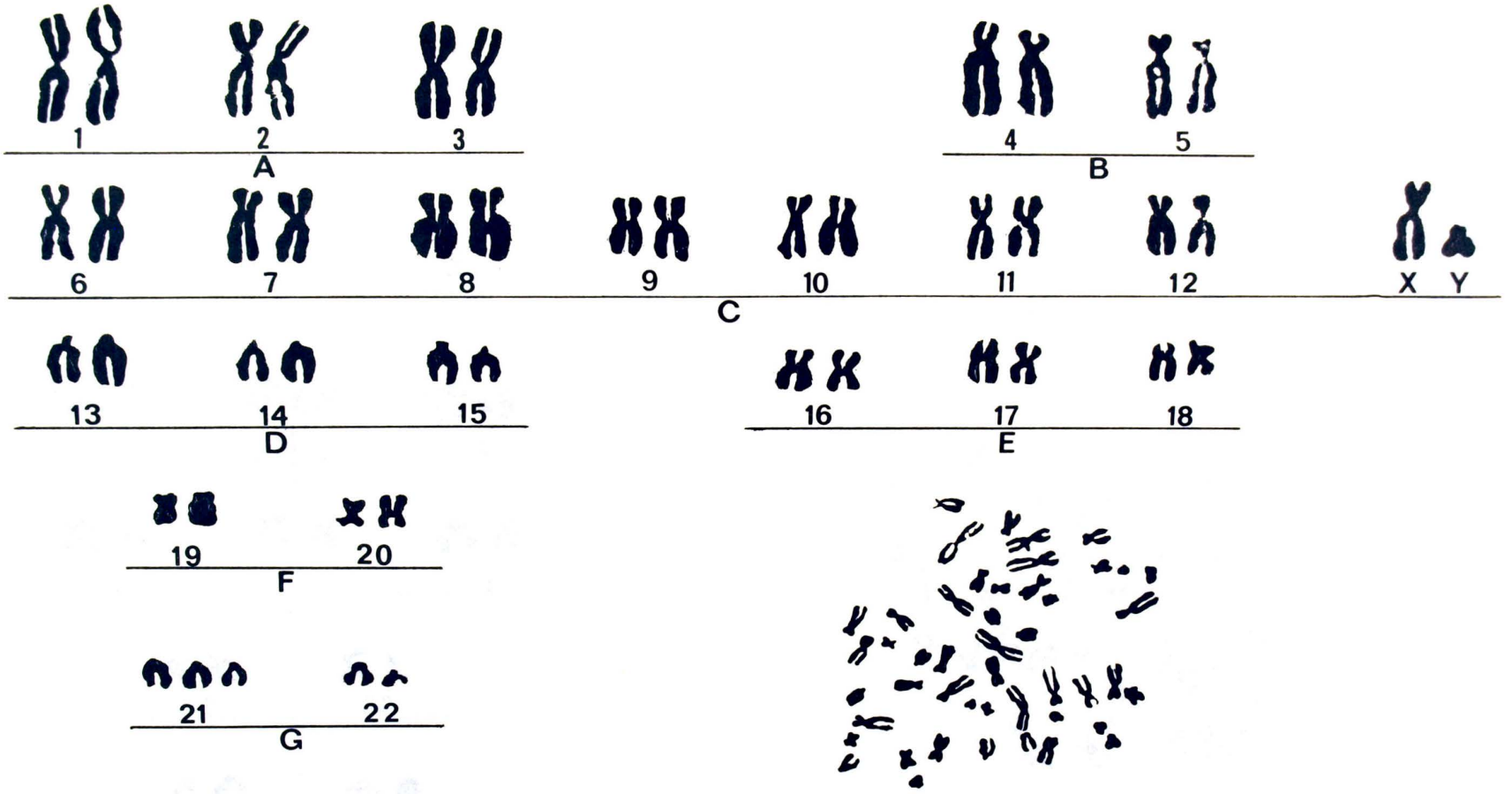

Figure 3. Karyogram Showing Trisomy of Chromosome 21 From a Patient with Typical Down's Syndrome.

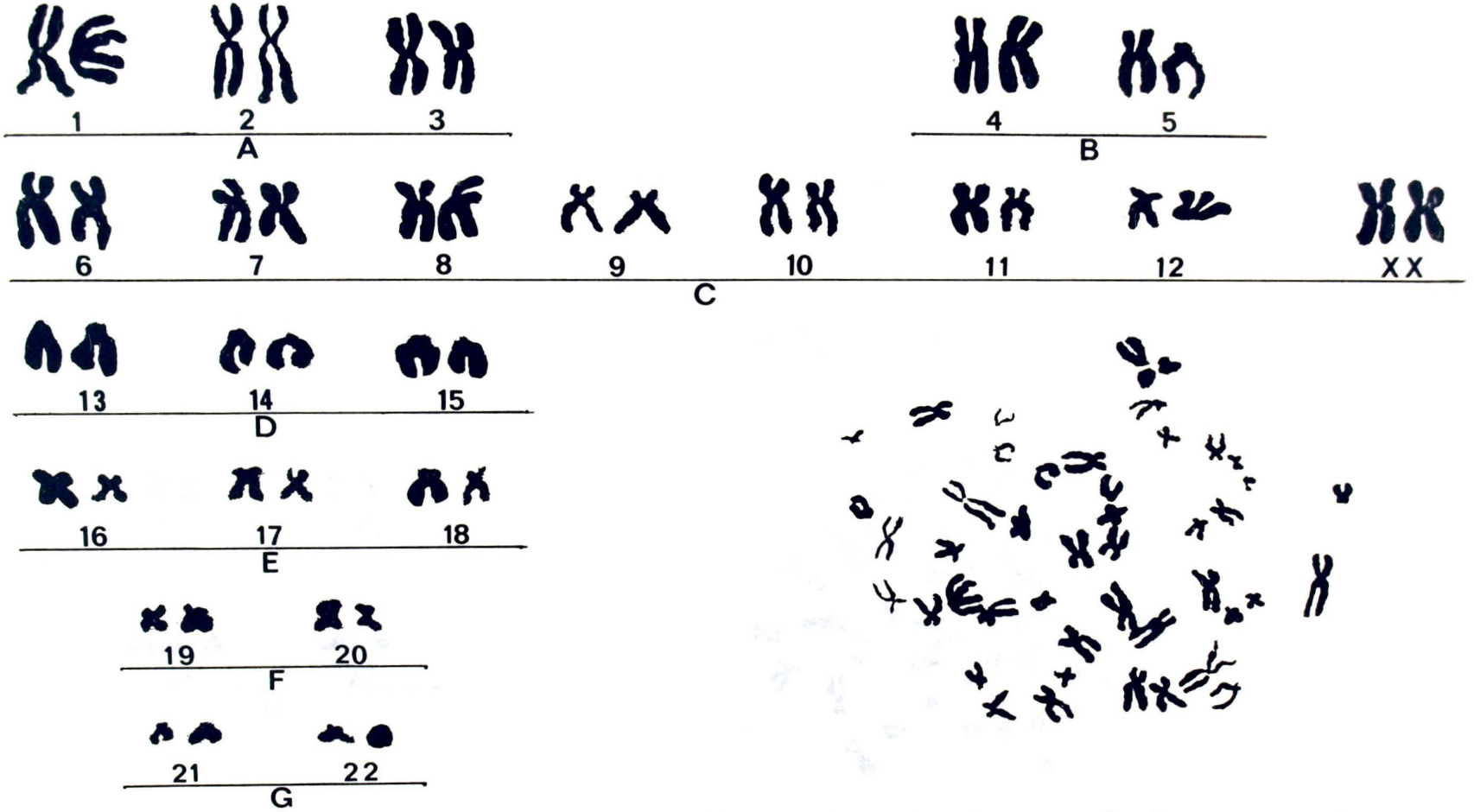

Figure 4. Karyogram with the Deletion of a Portion of the Short Arm of a B Group Chromosome (Chromosome 5).

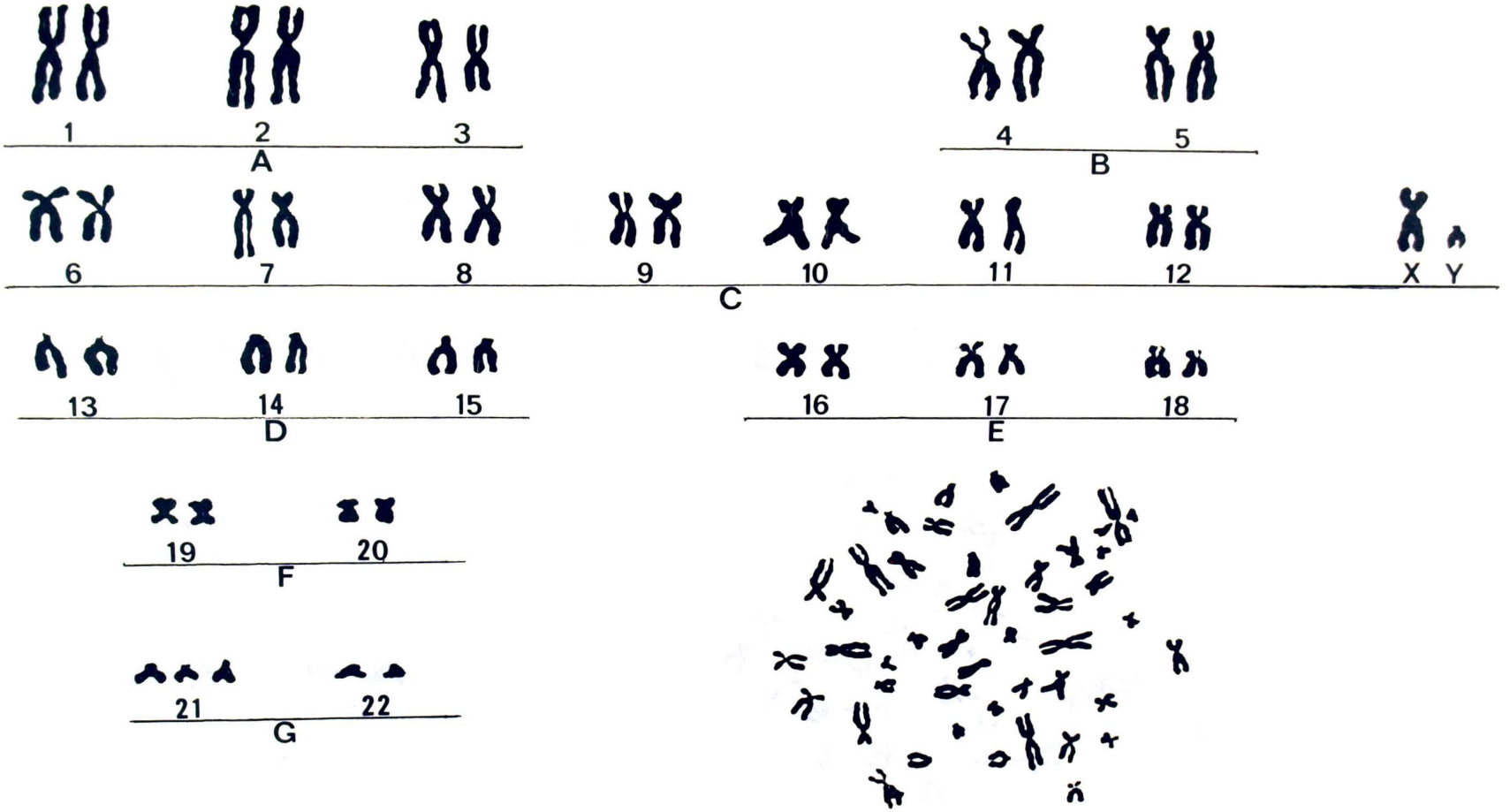

Figure 5. Karyogram of a Mongoloid Male with the Typical G Group Trisomy.

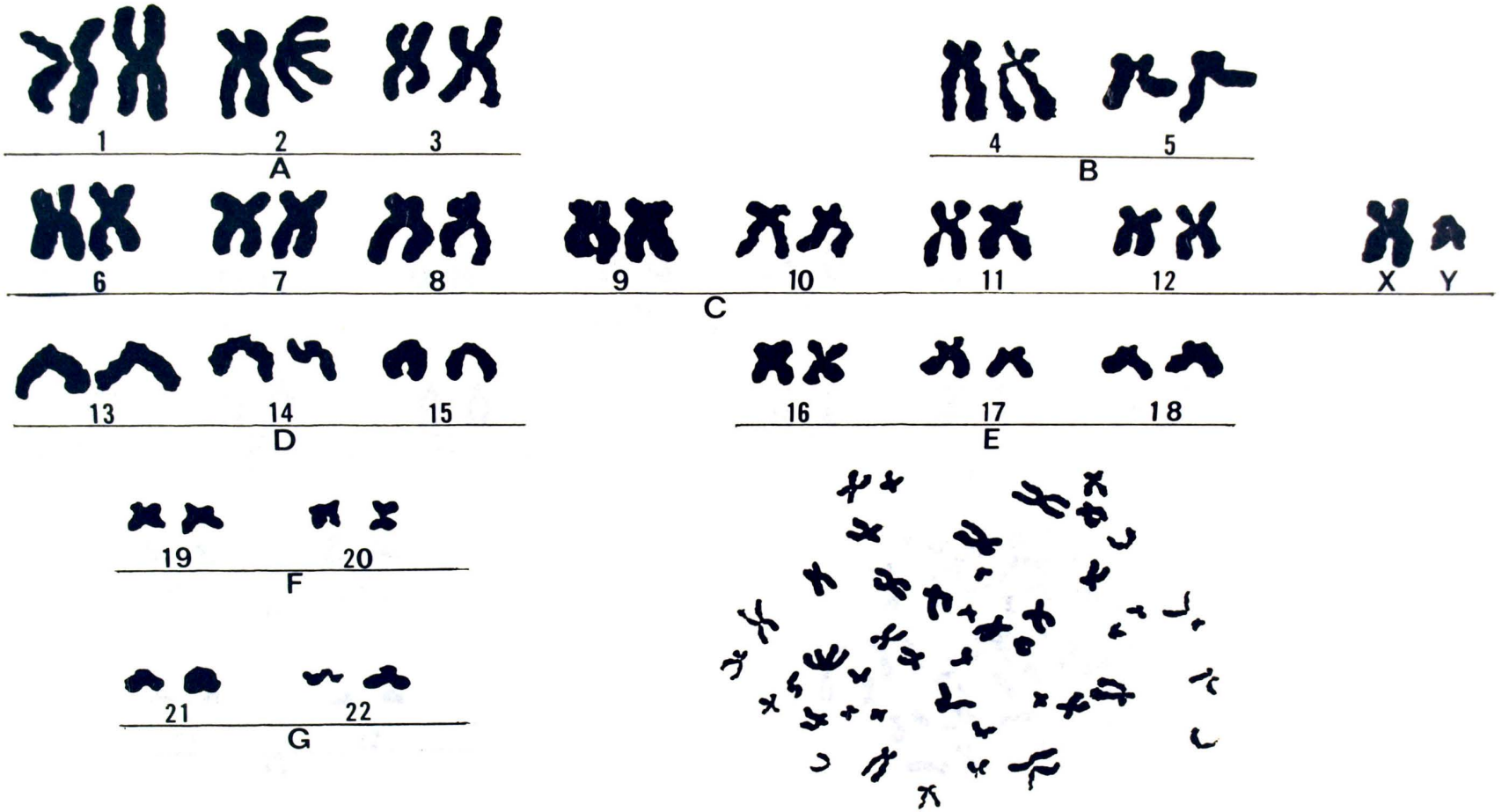

Figure 6. Normal Karyogram of a 23 Year Old Male (Father of the Down's Syndrome Male from Figure 5).

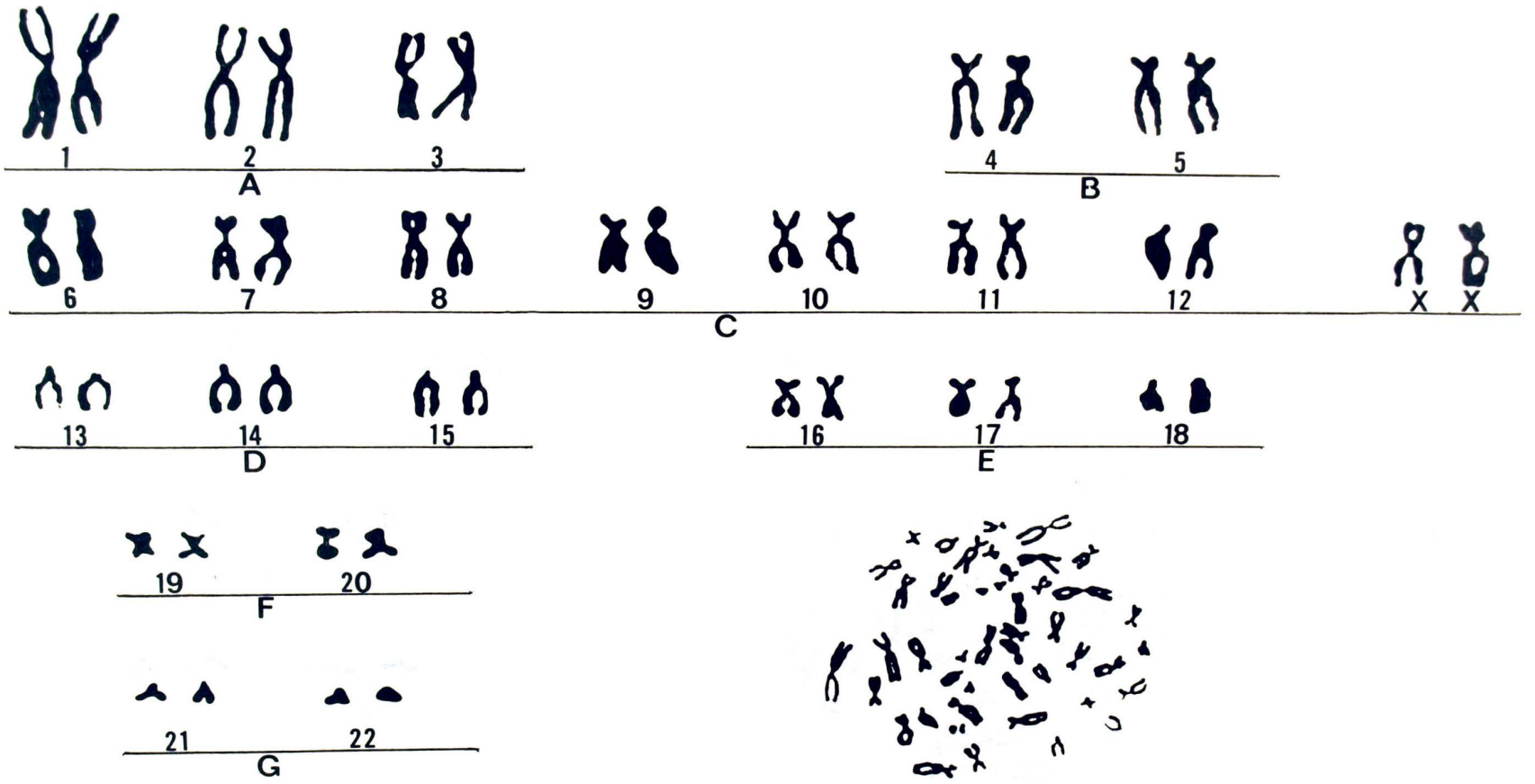

Figure 7. Normal Karyogram of Mother of Mongoloid Male from Figure 5.

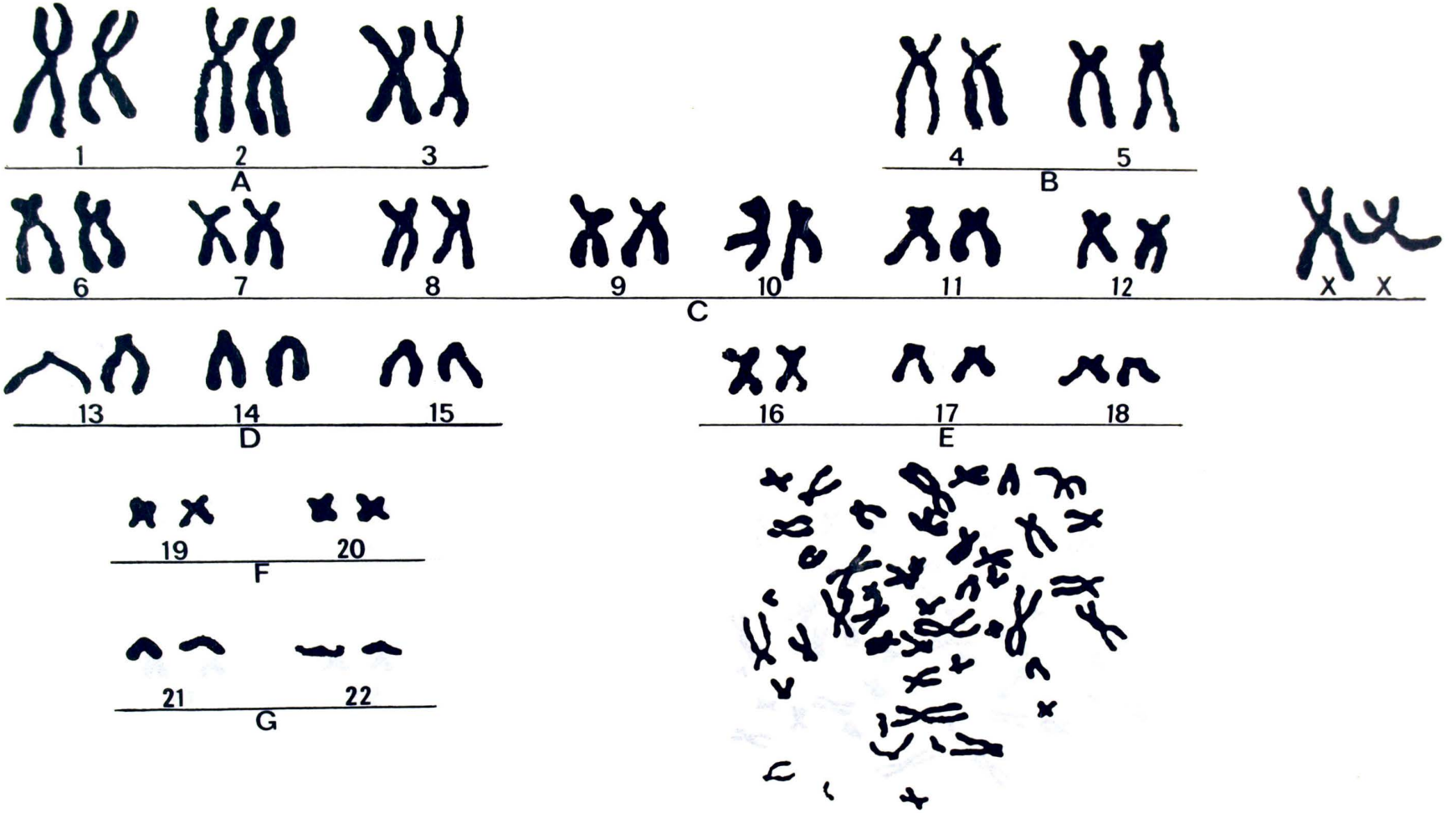

Figure 8. Normal Chromosomal Complement of 21 Year Old Female (Same Patient as in Figure 7).

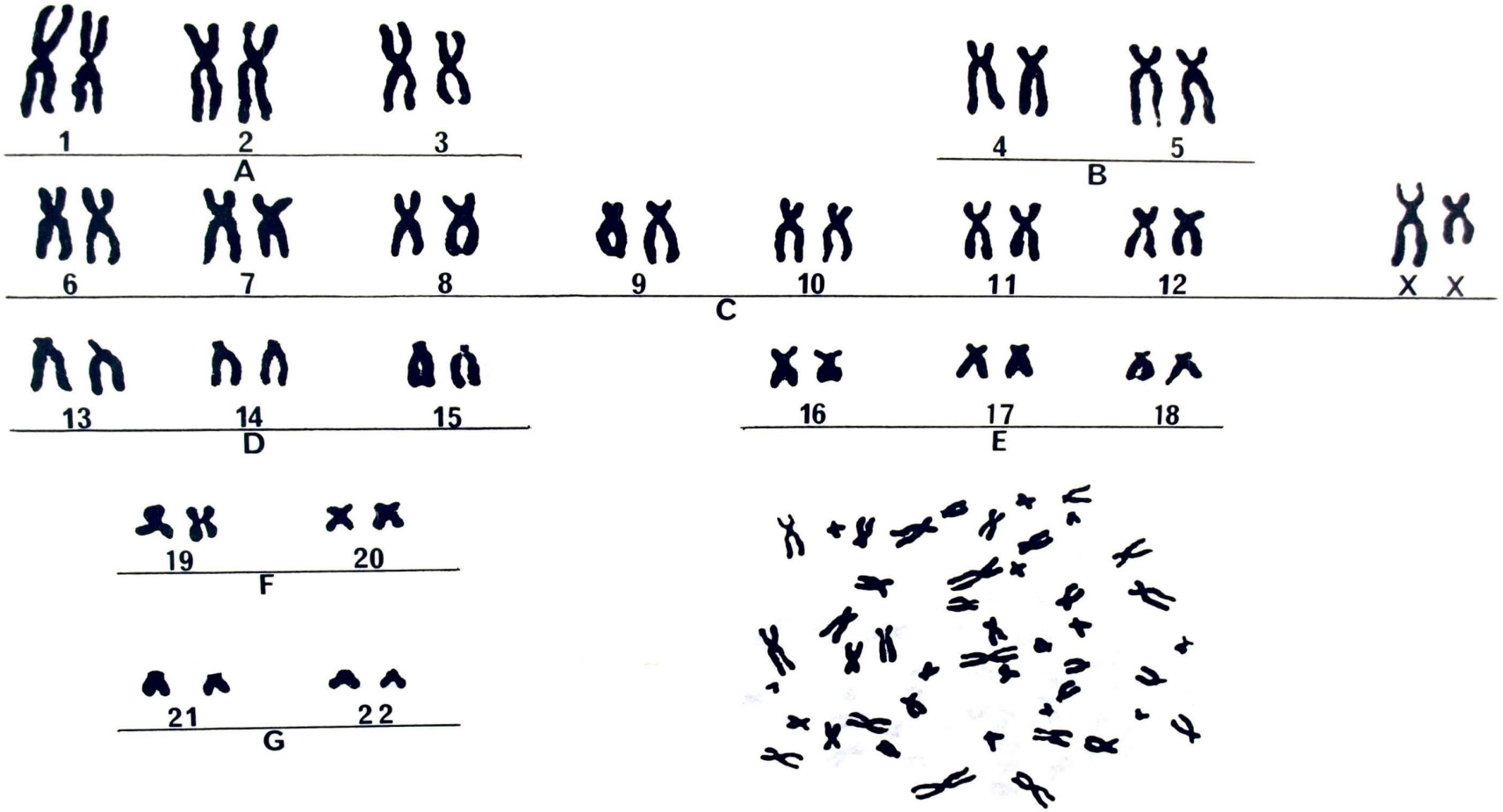

Figure 9. Apparently Abnormal Karyogram of the Same Female Shown in Figure 7 & 8. Note the Mispaired Chromosomes Identified as the "X".

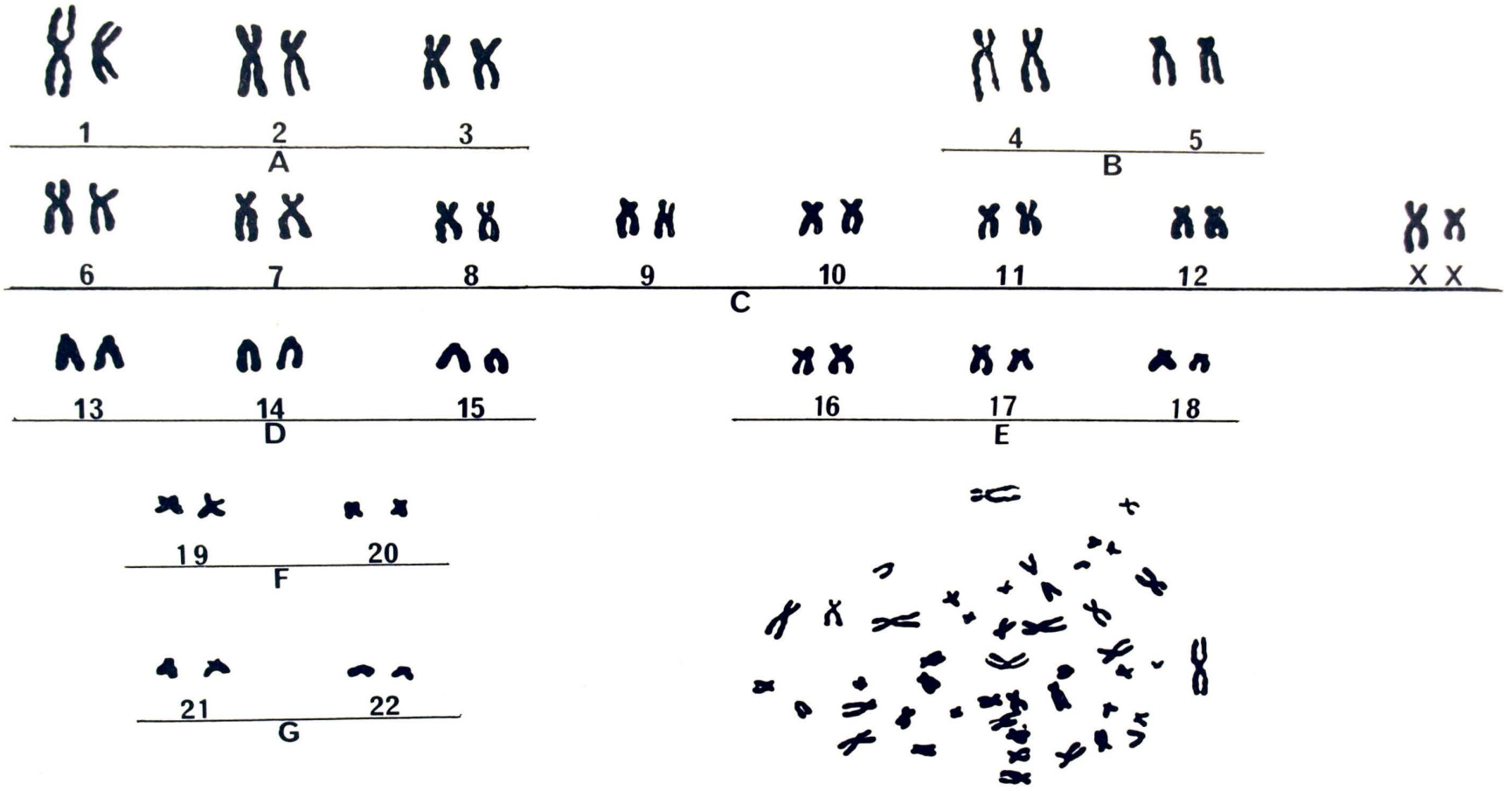

Figure 10. Abnormal Karyogram with Two Non-Paired Chromosomes Identified as the X Pair (Same Patient as in Figure 9).