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CYTOGENETICS AND FANCONI'S ANEMIA: EXPERIMENTAL AND OTHER STUDIES OF A FAMILY

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CYTOGENETICS AND FANCONI'S ANEMIA: EXPERIMENTAL AND OTHER STUDIES OF A FAMILY

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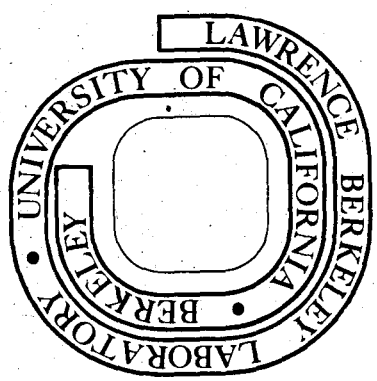

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Cytogenetics and Fanconi's Anemia:
Experimental and Other Studies of a Family

William D. Loughman

ABSTRACT

The genetic and cytogenetic literature pertaining to Fanconi's Anemia (FA) is reviewed. A case is made against the simplistic view of FA etiology. Physical and clinical data aregiven for a case of FA, her living family, and three deceased FA sibs. A pedigree of 188 individuals in 7 generations is discussed: The incidence of leukemia and diabetes is unremarkable, but infant mortality is elevated, reaching 21% in the paternal kindred.

Experimental studies span 5 1/2 years from diagnosis to termination of treatment. FA bone marrow cells contained no chromosome abnormalities. Cultured FA lymphocytes contained chromatid exchanges, gaps, and breaks, with multicentric chromosomes but virtually no other aberrations. Chromatid exchanges were more common initially than chromatid breaks; this ratio was reversed after 5 1/2 years. The chromatid exchange frequency declined exponentially with a halftime similar to the halflife of lymphocytes, and concordant with the period of clinically effective treatment. Multicentric chromosomes declined in frequency through the course of

treatment and were less frequent than chromatid exchanges. Simple chromatid breaks did not decline in frequency. Homologous chromosomes were involved in exchanges somewhat more frequently than were non-homologs; long chromosomes were involved more frequently than short. Cells with endoreduplicated chromosomes were uncommon, but increased at the termination of therapy. Other family members' chromosomes were normal.

Mitotic index determinations on cultured FA lymphocytes indicated either multiple cell populations or a cell cycle disturbance. In vitro survival of FA lymphocytes was below normal and biphasic after 48 hours of culture. Some reduced survival was spurious, and indicated a cell membrane defect.

Cultures of fresh normal lymphocytes with FA plasma did not show increased chromosome aberrations. Older cultured normal lymphocytes in conditioned FA medium displayed a seven-fold increase in chromatid breaks.

Electron micrographs of fresh FA lymphocytes were not different from normal. Micrographs of cultured FA lymphocytes displayed unusual lacunae in the endoplasmic reticulum, reduction in ribosome numbers, an increase in granules thought to be glycogen, and large mitochondria with unusual cristae.

Cell cycle analyses were performed. The mother's

G_1 phase probably was prolonged; the durations of either or both of S and G_1 were variable in both parents. Cell cycle phases of the normal sib were the same as in controls. The FA cell cycle times were not determined: a large fraction of her dividing cells failed to label with radiothymidine. A smaller fraction of both parents' cells failed to label. This phenomenon is suggested as the basis of a simple test for the FA carrier condition. A small fraction of the mother's cells labelled late in G_2. Only the FA case and her mother had a small fraction of intensely labelled cells. Normally-labelling FA cells were considered to consist of two populations, one with an abnormal cell cycle.

Hexokinase and thymidine kinase activities were normal or elevated in blood cells of the FA case and her family.

Lymphocyte chromosome repair time during G_1 was measured in all family members; all were approximately 4 hours as in controls. The results in the FA case suggested an extreme dispersion of repair times, or the existence of multiple cell populations.

X-ray induced aberration rates during the first G_1 and the last G_2 of cultured lymphocytes were the same as controls in all subjects' cells.

Cytogenetic abnormalities in FA lymphocytes occur in a simple pattern inconsistent with chromosome fra-

gility and mis-repair. The "exchange" theory of chromosome aberrations is more in accord with the experimental evidence.

The action of a physical agent on FA chromosomes is inconsistent with the available evidence. The action of a virus is consistent with but not proven by the experimental evidence provided.

The recessive gene hypothesis of FA causation was re-examined; a more complex genetic basis was considered likely.

INTRODUCTION

Fanconi first described the somewhat enigmatic disorder which now bears his name in 1927 [34]. Since then only about 200 cases have been reported in the world's medical literature [140,141]. Despite the rarity of the condition, or perhaps because of it, a number of clinical reviews are available [27,35,44,73, 79,140,141].

Also known as congenital pancytopenia, or familial hypoplastic anemia of childhood, Fanconi's Anemia (FA) is a rare and usually fatal condition. It is characterized by its familial occurrence, a progressive and severe pancytopenia with bone marrow hypoplasia, and a spectrum of congenital anomalies. The latter may include some or all of the following: patchy brown skin pigmentation, short stature, strabismus, hypogenitalism, hypoplasia of the thumb and radius, hypoplasia of the kidneys and spleen, microcephaly, microphthalmia, mental and sexual retardation, and other less common symptoms. The pancytopenia and marrow hypoplasia usually develop within the first decade of life [27,35,140,141]. Death most often occurs before sexual maturity, and only a few adult cases are known [17,27,103,128]. Both sexes are affected, but there is a nearly two to one preponderance of male over female cases [35,44]. There is no evidence of predilection for occurrence in any ethnic group or geographi-

cal area [27,140].

For 25 years after Fanconi's original case report, there was an increasing but largely circumstantial belief that FA was due to homozygosity for a recessive gene. Then Reinhold et al, reviewing 21 cases of FA in 14 families, provided more substantial support for the hypothesis [98]. Using the "cast out the propositus" method of Haldane [48], they concluded the incidence of FA in families was "...compatible with... the recessive gene hypothesis." Subsequently Nilsson reviewed 22 families with 47 cases of FA among 125 sibs [79]. This ratio is compatible with recessive inheritance, and without statistical analysis Nilsson agreed with Reinhold et al.

This view is widely held now, but was disputed by a number of later workers. A series of 117 cases was reviewed by Weicker [138] and Weicker and Fischel [139], who found a 1:1.17 ratio of affected to unaffected sibs. They believed a dominant gene was involved. In several single case reports, recessive inheritance seemed improbable: Imerslund described a mother with congenital abnormalities like those in FA who gave birth to a typical FA child [60]; she considered dominant inheritance likely. O'Neill and Varadi reported an FA child born to an FA mother [87]. Hoefnagel et al reported typical FA in two cousins, and expressed doubts about recessive inheritance [55].

Fanconi himself has suggested a dominant gene may be involved in FA [35]. Congenital anomalies, hematological symptoms, or chromosome aberrations (discussed below) have been observed in non-anemic relatives of FA cases [44,55,91]. Van Buchem et al. described three FA sibs; one gave birth to a child with severe congenital abnormalities who died shortly after birth [17].

These difficulties and others have been reviewed by several authors: Schroeder and Kurth are led to believe the diverse symptoms of FA, including effects in non-anemic cases, are due to a "weakened constitution" which is determined genetically in part [116]. Fanconi considers them the result of "intermediate" inheritance and "extreme pleiotropy" [35]. Dawson has suggested the diversity of FA symptoms could be the result of "variable gene expressivity" [27]. German surmised that FA may be genetically heterogenous [41].

Several authors have invoked external factors acting on a susceptible genotype to explain the varied congenital anomalies and the delayed onset of pancytopenia in early childhood. Among others, Schroeder and Kurth suggest as yet undiscovered agents may be affecting susceptible individuals [116]; while Swift and Hirschhorn have proposed viral infection as the causative agent [128].

FA appears to predispose its sufferers and even their relatives to risks other than anemia. Garriga

and Crosby demonstrated a markedly increased incidence of leukemia in FA patients and their near relatives [37], a finding generally confirmed by Bloom et al. and by Schroeder and Kurth [11,116]. Swift showed an increased risk of malignant neoplasm among presumptive heterozygotes for the FA gene [127]. More recently Swift et al. showed a six-fold increase in the incidence of diabetes mellitus among female, but not male, FA heterozygotes over that found in an age-matched control group [129].

Cytogenetic studies in Fanconi's Anemia have been most interesting: good but selective reviews have been provided by Bloom et al. [11], German [41], Schmid [108], and Schroeder and Kurth [116]. In 1964 Schroeder demonstrated the important fact that cultured blood leukocytes from FA cases showed a high frequency of chromosome abnormalities [115]. This observation was confirmed by Schmid et al. [109]. Along with Bloom et al. [11], they could find no chromosome abnormalities in bone marrow cells. Disputing the latter finding, Swift and Hirschhorn found chromosome abnormalities not only in marrow cells, but also in cultured skin cells in addition to cultured blood leukocytes [128].

The often remarkable display of chromosome aberrations in cultured FA cells led many authors to write of the "spontaneous breakage" [116], the "increased

instability" [41], or the "susceptibility" to chromosome breakage [35,128] of FA chromosomes. Higurashi and Conen provided formal support for these ideas when they found a 2- to 4-fold increase over control values in the radiation-induced chromosome aberration rate in cultured FA cells [54]. Schuler *et al.* also found increased aberration rates in the chromosomes of FA cells treated with an alkylating agent [117].

A number of investigators have remarked upon the predominance of chromatid-type over chromosome-type aberrations in cultured FA cells [109,112,114,115]. Bloom *et al.* have found no chromosome-type aberrations in cultured FA tissue [11]. Schmid considers the chromosome-type aberration to be little, if any, more frequent in FA than in normal cells [108]. Chromatid exchanges in normal cells are very rare, being present in about 0.02% of all cells counted [69,70,108]. Yet this type of chromatid abnormality is among the most common found in FA cells, sometimes being present in more than 25% of the cells [108]. Coupled with the observations of Lohr *et al.* [65,66], this led Schroeder to propose a chromosome repair defect arising from a hexokinase deficiency in FA. She proposed two forms of FA, with and without deficiency of cellular hexokinase, and with differing patterns of chromosome abnormalities [113,114,116]. Schmid considers the chromatid exchanges seen in FA cells to be *a priori* proof

of the existence of mis-repaired simple breaks; he thus implicitly denies the existence of a quantitative repair defect [108]. In reviewing their own and others' cytogenetic investigations, Bloom et al. concluded that chromatid exchanges, in FA cells at least, arise from an undiscovered mechanism distinct from simple misrepair of pre-existing chromatid breaks [11]. Earlier, German [39] and German and Crippa [43] had cited the phenomenon in FA as evidence of somatic crossing-over.

A number of authors have reported increased endoreduplication in cultured FA cells [11,55,91,108,115, 117]. Schmid, however, considers this to be very uncommon and not at all characteristic [108].

It is difficult to relate the chromosome aberrations found in lymphocytes to the myeloid cell pancytopenia so characteristic of FA. The marrow, whose cells show little or no chromosome abnormalities, is markedly hypoplastic; no known pathology is associated with the lymphoid cells or lymph nodes [27,35,44,140, 141].

The possible role of chromosome aberrations in causing both congenital defects and leukemia in FA has not been overlooked, nor has the potential role of viruses in causing all three phenomena [116,128]. And yet, Schmid among others believes the chromosome damage is symptomatic and secondary to an unclarified

cell-lethal process [108].

Summing all the foregoing makes apparent some disagreement in both the genetic and cytogenetic evidence bearing on FA. The majority of reports have been descriptive or observational; only a few describe experimental studies designed to clarify the fundamentals of FA etiology. Most experimental studies are in need of either confirmation or extension. Long term consecutive studies of FA individuals, more extensive family studies, and further experimental studies are needed.

This report presents experimental studies and observations spanning the 5 1/2 year survival of a case of FA, with concurrent studies of her parents and only surviving sib. The experiments were designed to test hypotheses derived from observation, and in turn they lead to new hypotheses of the etiology of FA. Experiments bearing upon the nature and origin of the chromatid exchanges seen in FA are described. Evidence is presented for alterations of cell membrane permeability and the cell cycle in cultured FA lymphocytes. The data presented are compared with previously published material, and with the various hypotheses advanced by earlier authors.

REPORT OF A CASE OF FANCONI'S ANEMIA

The Propositus

Patty H., a 14 1/2 year old Caucasian female born in April 1958, is this study's propositus. She is the second daughter and the fourth of five children born to normal parents of Irish-English-Welsh ancestry. Two younger brothers and probably a later sister, all described below, succumbed to FA. An older sister remains physically and hematologically normal.

Patty's birth and earliest childhood were unremarkable. She contracted and recovered from many of the usual childhood illnesses without sequelae. At age 8 she appeared physically normal, and hematological and other studies performed then revealed nothing unusual.

First clinical signs of illness were noted in mid-1967, following a year of severe upper respiratory infections. A definite diagnosis of FA was made in October 1967; the child was then 9 years old. Treatment, described below, was begun at this time.

At the present (January 1973) Patty has a below-normal stature. A skeletal survey showed no abnormalities, except for unusually short middle phalanges in the fifth fingers of both hands. The child's skeletal age is normal. Her eyes are small; her skin has a yellow-tan cast, with a few rounded brown macular areas (cafe-au-lait spots) on her chest and arms. Apart

from these, there are none of the other physical stigmata of FA. She is of average or higher intelligence, and she had been active in school and socially. Menstrual periods and breast development appear to be normal, following a delay which may have been occasioned by recently discontinued androgen therapy.

Initial clinical findings included marked reduction in all formed elements of the blood except lymphocytes, and a hypocellular bone marrow. Slow clinical improvement was obtained with only hematinic therapy (folic acid, vitamin B_{12}, pyridoxine). This became ineffective in February 1968, following a relapse apparently prompted by an upper respiratory infection. Although adrenal steroid therapy (Prednisone) was begun in March 1968, the child's clinical condition did not improve. Transfusions of whole blood and platelets, with administration of antibiotics and gamma globulin, became necessary. Experimental endosteal curettage was performed in October 1968, without significant effect [136]. As a last resort, androgen therapy (Delatestryl, then later Halotestin) was begun in December 1968. The hematological response was not marked, and transfusions continued to be necessary. Masculinization with hirsutism, deepened voice, acne, and other changes resulting from androgen therapy were marked within four months. At this time Patty's peripheral blood showed hemoglobin levels in the 9 grams-

percent range, maintained largely by transfusion. White blood cell counts, largely composed of lymphocytes, were 2200/mm^3. Platelets were 40,000/mm^3, and the bone marrow remained markedly hypoplastic.

In an attempt to minimize the masculinizing side-effects of androgen therapy, treatment was changed in April 1970 to an experimental androgen (oxymetholone, Syntex Laboratories). Unexpectedly, hemoglobin rose to normal levels (13.9-15.6 grams-percent), and white blood cells and platelets tripled. This good hematologic response continued for 2 1/2 years, but was not accompanied by remission of masculinization.

In September 1972, with the still severe and distressing masculinizing effects of the androgen therapy uppermost in her mind, Patty insisted on discontinuing all medication. She was fully aware that this could cause reappearance of her disease, or even death. After extensive consultation with the parents, the child, and medical experts, the attending physicians acceded to Patty's wish.

Four months later, in January 1973, the child exhibited a decrease in facial hair. A long-standing problem with insomnia disappeared, and she continued to feel well. Her very first two menstrual periods occurred, exactly 28 days apart. Tenderness over the breasts and some breast tissue growth was noted. Hemoglobin levels had fallen to 11 grams-percent, white

blood cells were down to 2900/mm^3, and platelets were reduced to 40,000/mm^3. At this time Patty H. has survived with FA for five and one-half years.

A 5 1/2-year summary of hematological parameters for Patty H. is shown in Figure 1. The Figure also shows the schedule of drug therapy, and the dates on which were obtained the blood and marrow samples pertinent to the data given in this report.

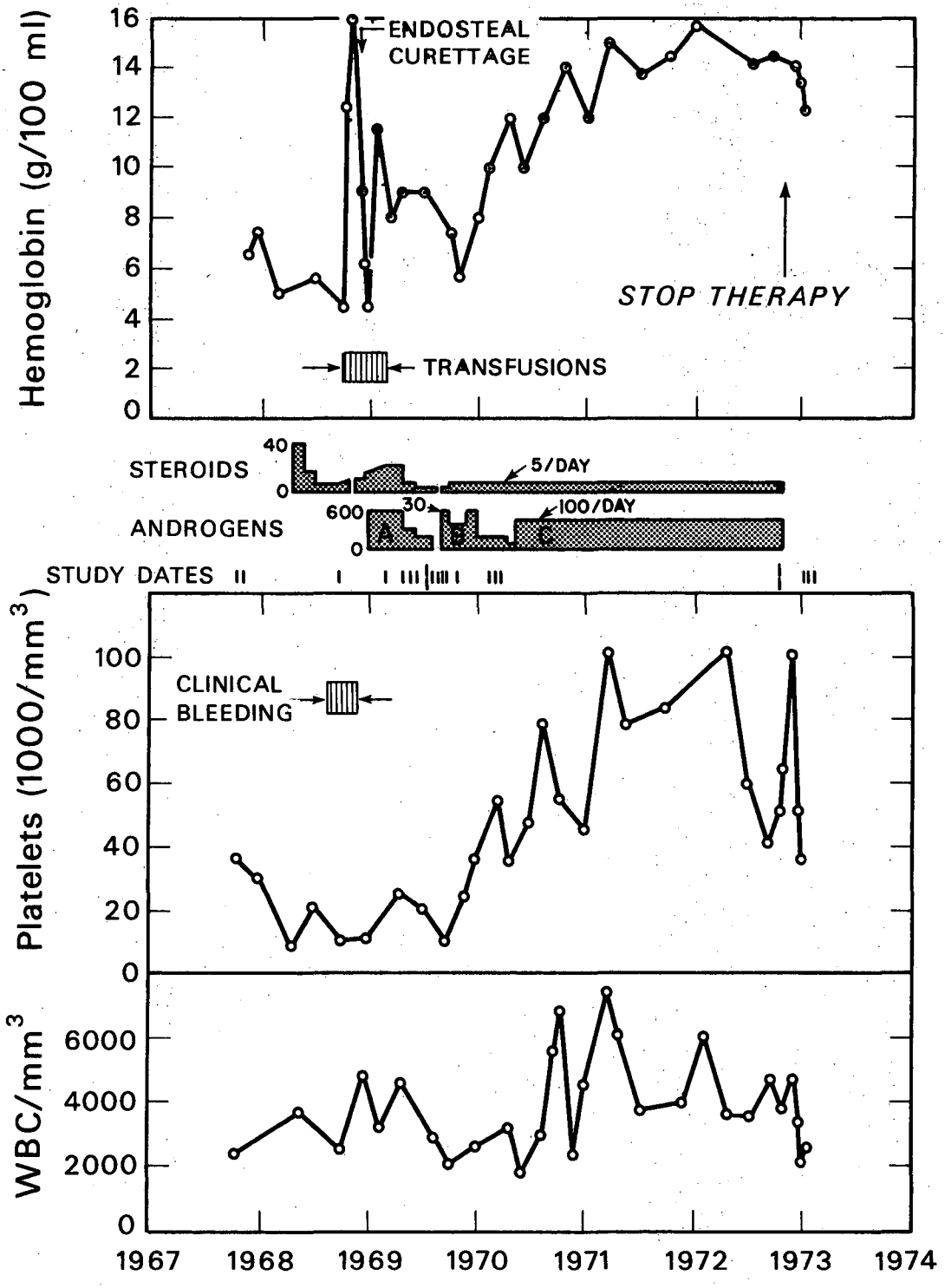
The Family

Cathy H., born uneventfully in March 1954 and now 19 years old, is the family's first child. She contracted the usual childhood diseases, recovering from them without sequelae. At present, Cathy is physically and hematologically normal, with at least average intelligence and normal sexual development.

John H. is the family's second child and first son; he was born in August 1955 when his mother was 38 years old. At birth he had a reduplicated right thumb but no other congenital abnormalities. This was corrected surgically in February 1957, at which time he was hematologically normal. Anemia was first noted in 1959, when he was four years old and after a bout of measles. One year later in November 1960, after a largely unsuccessful treatment regimen of hematinics and transfusions, a diagnosis of Fanconi's Anemia was given. Prednisone and androgen (Halotestin) therapy

Figure 1. Clinical parameters of the propositus: 5 1/2 year summary.

Steroids in mgs per day. Androgens in mgs per day: A = Delatestryl; B = Halotestin; C = oxymetholone. Note that the improved platelet counts and hemoglobin levels were coincident with Halotestin therapy, but WBC improved coincident with oxymetholone treatment.


Hemoglobin (g/100 ml)
16
14
12
10
8
6
4
2
0
ENDOSTEAL CURETTAGE
STOP THERAPY
TRANSFUSIONS
STEROIDS
40
0
5/DAY
ANDROGENS
600
0
30
100/DAY
A
B
C
STUDY DATES
Platelets (1000/mm3)
100
80
60
40
20
0
CLINICAL BLEEDING
WBC/mm3
6000
4000
2000
0
1967
1968
1969
1970
1971
1972
1973
1974

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was instituted, without encouraging results. Further transfusions were necessary, but by April 1963 he required hospitalization for cutaneous and internal hemorrhage. He did not respond to heroic therapy, and died in April 1963 at the age of 7 1/2 years, a bit more than 3 years after the diagnosis of FA.

David H., the third child and second son, was born in December 1956. His birth and very earliest childhood were uneventful. David's growth was slow; by 5 years of age he was unusually short in stature and below normal in weight. He was microcephalic with a degree of mental retardation. Strabismus was present. The middle phalanges of the fifth fingers of both hands were abnormally small; his bone age was retarded. The child's skin was of a markedly darker cast than was found in other members of the family, and this was exaggerated in the perigenital and anal regions and irregularly around the neck and over the trunk.

David enjoyed generally good health until September 1961. Over a period of several months he had a number of infections and illnesses including chickenpox. These were followed by a series of "not serious" upper respiratory infections. During this time, his appetite fell off, he was noticeably pale, and his parents brought him to medical attention. A diagnosis of FA was made in February 1962, following clinical exam-

inations which revealed anemia, pancytopenia, and hypoplastic bone marrow.

Over the next two years treatment consisted of Prednisone, and then also testosterone. Splenectomy was performed. All therapy was without significant effect; the child's condition declined and transfusions were necessary. In February 1964 he suddenly developed acute gastroenteritis and died at the age of 7, almost exactly two years after the diagnosis of FA. An autopsy verified the cause of death, and revealed in addition atrophic adrenal glands and a significantly enlarged heart.

Marjean H. was the last child born to the parents. Her birth in November 1959 was normal, but she died shortly after birth with multiple congenital anomalies. These included: encephalocoele, "slant" eyes, absent external auditory canals, syringomyelia, microgyria, cerebellar agenesis, a supernumerary thumb on the right hand, and a prehensile thumb on the left hand. No hematological data were available for Marjean, but she was said to be exceptionally pale at birth.

The family has adopted a daughter aged 7 years. Margaret H. was born in April 1966 and has been a well child. Not included in this study, she is mentioned here only for completeness.

Ellen H., aged 55 years, is the mother of the

five sibs described above. She was born uneventfully November 1917, the second child and only girl in a sibship of three. Aside from a long-since relieved episode of "Bright's disease" in childhood, her early life and young adulthood were unremarkable. She contracted diabetes in April 1971, two years after her last child was born. This has been treated with insulin and Diabinase. Apart from this, Ellen is physically, hematologically, and intellectually normal. Her 53 and 57 year old brothers are living and well. Ellen's father died aged 32 in the 1920 influenza epidemic; her mother died at age 46 post-operatively after goiter removal. Both Ellen's parents are from large families: her paternal grandfather is a progenitor of "...one of the largest families in Utah..." (University of Utah records). Ellen's first child (Cathy) was born when she was 37 years old; her last (Marjean) when she was 42. She has had no miscarriages or abortions. A housewife and mother most of her adult life, Ellen has had no exposure to excessive radiations or hazardous chemicals. She has not received unusual medications.

Harold H., aged 46 years, is the father of the five sibs described above. He was born in April 1926, the third child and third of four sons in a sibship of five. Following an uneventful birth and childhood, he has enjoyed good health throughout his

life. Harold is physically, hematologically, and intellectually normal. A younger brother died at age 1 1/2 years of complications following measles and pneumonia; the other sibs are alive and well. Still living at age 82, Harold's father became "mildly diabetic late in life", and his mother is alive and in good health at age 75.

Like his wife, Harold has had no unusual medications or X-ray treatments. For many years employed as a railroad office worker, Harold has no known exposure to other radiations or hazardous chemicals.

The Kindred, and a Pedigree

Mr. and Mrs. H. are of the Mormon faith (Church of Jesus Christ of the Latter Day Saints); with their help access was gained to the remarkable records of the Church in Salt Lake City, Utah. From these records and additional information obtained directly from family members, a pedigree of the H. family and its kindred was constructed. This is shown in Figures 2 and 3.

Other than in direct line of ascent from the propositus, marriage partners are not shown. Ascertainment of births and deaths is complete within four of the seven generations depicted. Miscarriage and abortion is not indicated; this information has proved very difficult to obtain. As shown, the kindred contains

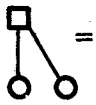
Figures 2 and 3. Pedigree of the kindred.

□ = male; ○ = female; ◇ = sex unknown.

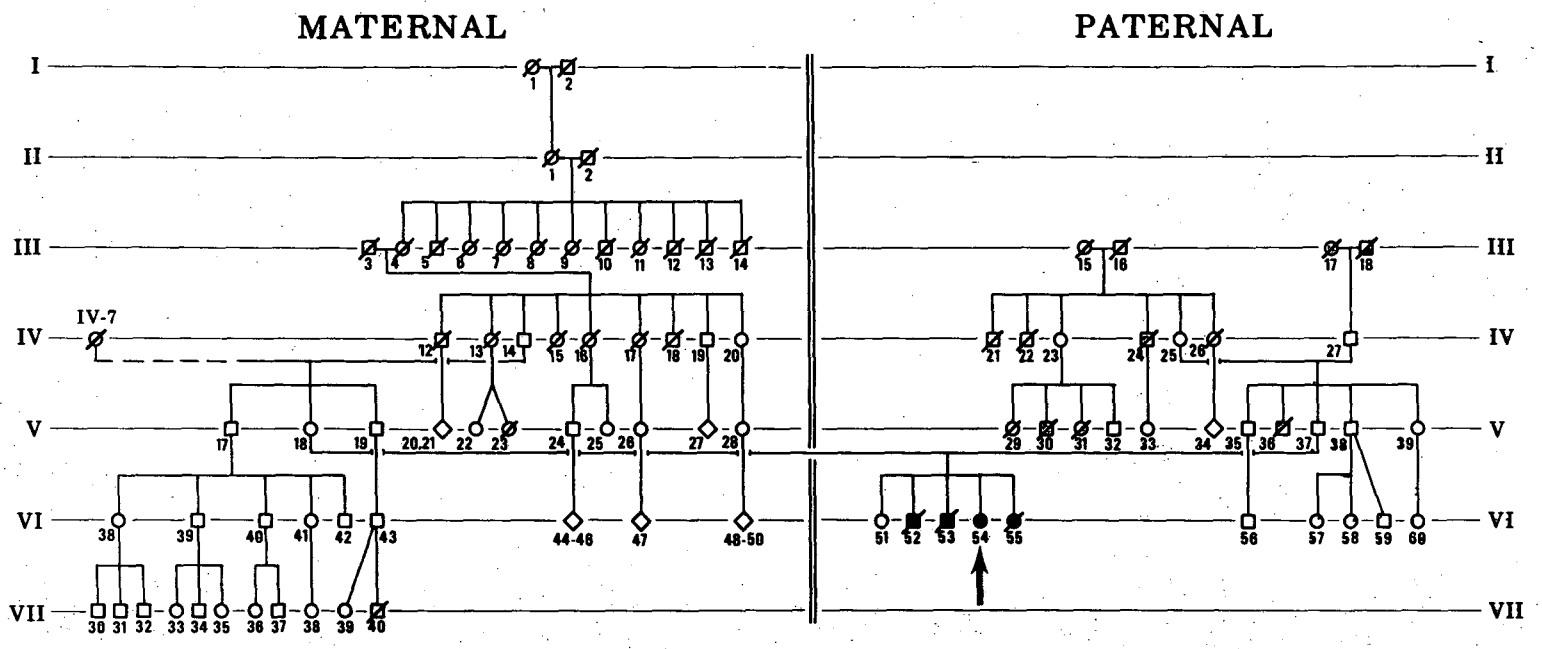
⌀ = deceased.

 = The propositus of this study.

 = twins, zygosity not known.

 = children from multiple marriages.

Ascertainment of births and deaths is complete in generations IV-VII. Individuals III-3 through III-14 all had large families. Individual III-3 had several wives, one of whom (alive at age 104) is the progenitor of one of Utah's largest families. Other than in direct line of ascent from the propositus, and in the primal generations, marriage partners are not shown.


MATERNAL
PATERNAL
I
II
III
IV
V
VI
VII
IV-7
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MATERNAL (cont.)

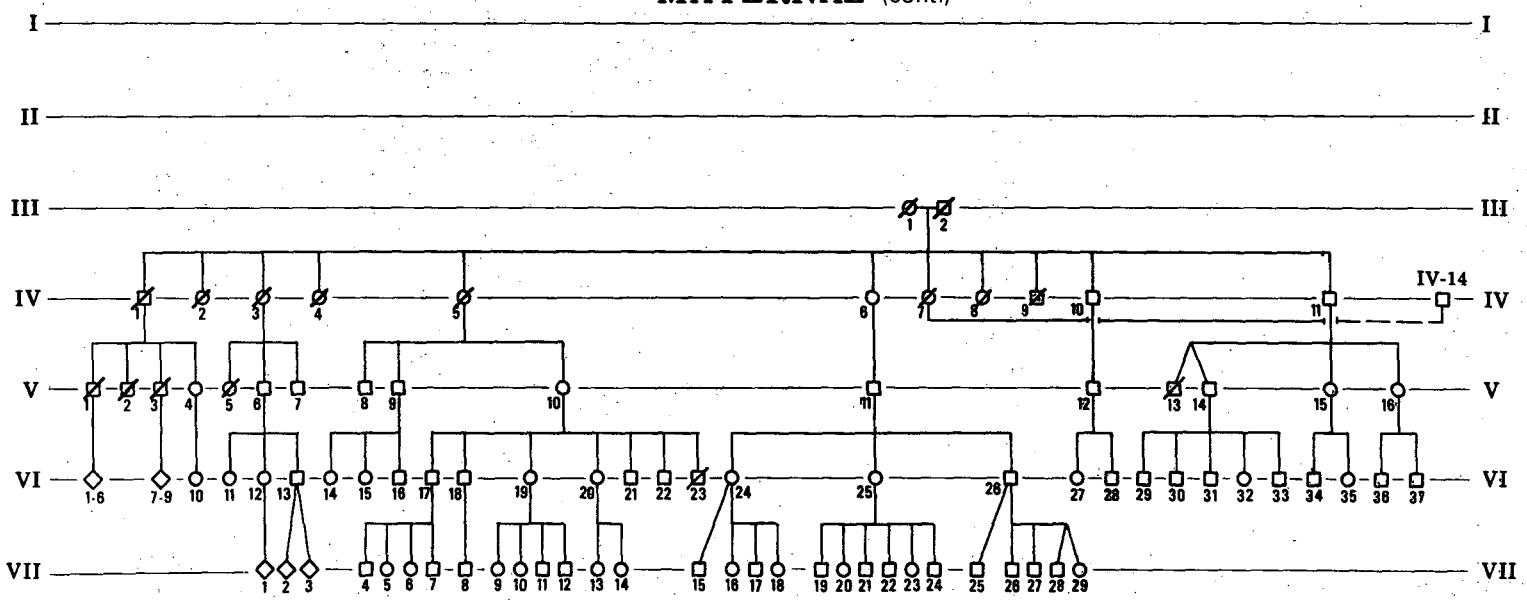

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Table 1. Infant death, neoplasia, diabetes, and FA in the kindred

Subject	Sex	Age at death	Cause of death/comments
		INFANT DEATH	
IV-2	F	2 days	Unknown
IV-8	F	"infant"	Unknown
IV-9	M	1 year	Unknown
IV-15	F	at birth	Unknown
IV-18	M	at birth	Unknown
IV-21	M	"infant"	Unknown
IV-22	M	"infant"	Unknown
V-5	F	6 months	Failure to thrive; infection
V-13	M	1 week	Unknown; "very pale"
V-30	M	1 year	Possible viral respiratory infection
V-31	F	"few days"	Intestinal hemorrhage
V-36	M	1 year	Complications of measles
VI-23	F	at birth	Stillborn
VI-55	F	2 days	Fanconi's Anemia (see below)
VII-40	M	1 week	"Soft bones; pale; not developed"
		MALIGNANT NEOPLASIA	
IV-5	F	62 years	"Cancer"
IV-16	F	66 years	"Cancer"
V-1	M	"aged"	"Stomach cancer"
		NON-MALIGNANT NEOPLASIA	
IV-23	F	--	Ascites tumor, age 79; living at age 83.
VI-19	F	--	Benign breast tumor; living at age 20.
VI-20	F	--	Benign tumor of jaw; living at age 19.

Table 1. (Continued)

Subject	Sex	Age at death	Cause of death/comments
		DIABETES	
IV-7	F	47 years	Post-operative for goiter; diabetes "shortly before she died"
IV-27	M	--	Mild diabetic late in life; living at age 82.
V-18	F	--	Mother of FA propositus; living at 55 years.
V-35	M	--	Living at age 52.
VI-41	F	--	Diabetes: age 34; living at age 36.
		FANCONI'S ANEMIA	
VI-52	M	7 1/2 years	Acute internal and cutaneous hemorrhage.
VI-53	M	7 years	Acute gastroenteritis.
VI-54	F	--	Living at 14 1/2 years; survived with FA 5 1/2 years.
VI-55	F	2 days	Multiple congenital anomalies; "exceptionally pale".

188 individuals. Ascertainment of cause of death is complete for all 55 deceased members of the kindred; ascertainment of the condition of health of the 133 living members is incomplete but continuing. The medical status of nearly 70% of the living kindred is known with reasonable reliability at the present time (January 1973).

The paternal ancestors were Welsh who emigrated to Colorado, and Irish-Scotch who emigrated to Pennsylvania and thence to Colorado. From Colorado branches of the paternal kindred migrated to Nevada and California.

The maternal ancestors were English who emigrated to Utah late in the 19th Century. Since then branches of the family have taken up residence in several Western States, primarily in Utah and California.

It must be noted that the progenitors of this large kindred were all born in the British Isles; intermarriage at some time in the 19th century cannot be ruled out on the evidence presented. However consanguinity of any ancestors of the propositus is not shown through the three generations preceding her own.

Leukemia, Diabetes, and Infant Mortality

Garriga and Crosby first suggested an association between FA and leukemia [37], and the presumed association now is widely accepted [11,41,116]. Additional-

ly, Swift has reported an association between FA and non-leukemic malignant neoplasm [127]. Of the 55 deaths which have occurred in the kindred presented here, records certifying the cause of death show three individuals died of cancer. Eighty-two of the 133 living members are known to be free of neoplasia, but 3 have relatively benign tumors. The health of the 48 remaining living members is not known, but the incidence to date of neoplasia in this large kindred is not impressive.

Swift et al. have demonstrated an association between the putative FA gene and diabetes [127]. The records used to construct the pedigree given here usually do not show medical conditions which were noncontributory to death. Therefore the ascertainment of diabetes within the kindred is quite incomplete, often being based on verbal information from family members. At this time, with incomplete information, the incidence of diabetes in the kindred does not greatly exceed the 1-2% expected in the general population [56, 127]. Among the close relatives of the propositus, her mother, a maternal first cousin, and her paternal uncle and grandfather are or were diabetic.

With only scant comment on the observation, Nilsson reported an 8.8% perinatal mortality among the 79 "unaffected" siblings of FA cases which he reviewed [79]. In the FA kindred shown here, 14 of the

166 births in generations IV through VII, excluding the FA case, were infant deaths: an incidence of 8.4%. The incidence is most marked in the paternal kindred, where early postnatal deaths occurred in 21% of known births, not counting those in the study family. Social and economic conditions within the kindred have certainly varied with time and place, as must have also the quality and availability of medical care. These factors must influence the infant mortality rate; nonetheless the incidence of early postnatal mortality in this kindred seems altogether too high.

Table 1 lists kindred members known to have died as infants, those known to have had diabetes or neoplasia, and those known to have had FA.

INITIAL OBSERVATIONS, AND A HYPOTHESIS

Iron kinetic studies had been performed on David H. at the Donner Laboratory, University of California, Berkeley, during the course of his illness with FA. As a "follow-on" to that investigation, the propositus (Patty H.) became available to research physicians at the Laboratory. Initial cytogenetic studies were performed on Patty's lymphocytes, marrow cells, and fibroblasts as a pro forma adjunct to clinical examinations and iron kinetic studies.

Materials and Methods

Lymphocytes for culture were obtained in the following way: Peripheral blood obtained by puncture of the cephalic vein was aspirated into plastic syringes containing 50 I.U. heparin per ml of blood. After mixing by inversion, a fresh covered needle was attached to the syringe and it was allowed to stand needle-up in a rack at ambient temperature. Supernatant plasma (and white cells) exceeding 10% of the original blood volume was obtained after 1/2-hour of red cell sedimentation. After bending the syringe needle to enter the mouth of a small sterile screw-capped Erlenmeyer-type flask, the white-cell rich supernatant plasma was expressed into the flask by careful movement of the syringe plunger.

Two volumes of fresh tissue culture medium[1] were added to the flask, along with approximately 1/2-gram of finely divided iron powder[2]. The suspension was mixed gently by swirling, and the tightly-capped flask was placed in a shaker water bath at 37°C. The shaking cycle was adjusted to 100 strokes/min., with sufficient amplitude to just suspend the iron powder without foaming the fluid medium. After one hour the flask was removed from the bath, and a strong miniature Alnico magnet was held against its bottom for three minutes. Mature granulocytes and monocytes, having phagocytized iron particles, were magnetically removed from suspension. This procedure was necessary to insure consistency of lymphocyte behavior *in vitro* from experiment to experiment, and between subjects [68, 137]. After decanting the supernatant fluid, the magnetic cell-removal procedure was repeated, and a sample of the supernatant fluid was suitably diluted to obtain a cell count in a hemocytometer. This complete procedure is essentially that of Hastings *et al.* [50]. In earlier studies, the method consistently produced the best combination of high yield and high purity in comparison with other methods [68,96,121]. Lymphocyte

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[1]TC 199 or NCTC 109. Microbiological Associates, Albany, Calif.

[2]Iron Powder, Reduced Electrolytic Grade. Matheson, Coleman and Bell, Cincinnati, Ohio.

purity regularly was 95-100%, and absolute yields were above 50%. Principal contamination consisted of platelets and red cells; both were reduced orders of magnitude below their concentration in whole blood. Prior to culturing the cells, the suspension was concentrated by centrifugation, or diluted with both medium and fetal bovine serum (FBS)[1], to yield a concentration of 10^5-10^6 lymphocytes/ml, with 5% v/v autologous plasma and 5% v/v FBS. Although aseptic procedures and sterile glassware were used throughout the procedures described above, antibiotics were added to the cultures. Each ml of final culture medium contained 330 units of sodium penicillin[2], 170 μg of streptomycin sulphate[3], and 33 units of nystatin (an antifungal agent)[4]. As a mitotic stimulant, phytohemagglutin-M (PHA)[5] was added to a concentration of 2% v/v [84]. Five or ten ml aliquots of the lymphocyte suspension were planted in 15 ml screw-capped tissue culture tubes. These were placed in racks at a 45° slant, and with caps loosened they were placed in

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[1]Fetal bovine serum. Microbiological Associates, Albany, Calif.

[2]Cer-O-cillin. Upjohn Co., Kalamazoo, Michigan.

[3]Eli Lilly and Co., Indianapolis, Indiana.

[4]Mycostatin. E.R. Squibb and Sons, Inc., New York, New York.

[5]Difco Laboratories, Detroit, Michigan.

a darkened 37°C incubator. The incubator atmosphere was 5% CO_2 in air, with a relative humidity above 98%. Sixty-eight hours after initiation of cultures, mitotic arrest was effected by the addition of vinblastine sulphate[1] to a final concentration of 5-7 nanograms/ml [19,50,72].

Bone marrow cells for direct examination or for culture were obtained as follows: Under local anesthesia, the attending physician aseptically obtained 2-3 ml of bone marrow from the iliac crest by aspiration into a plastic syringe containing 500-1000 I.U. heparin. After rapid mixing by inversion, the specimen was divided into two unequal parts. The smaller part was placed immediately into a culture tube containing medium and also Velban. The larger part was diluted with medium and FBS to yield 10 ml aliquots having a packed cell volume of approximately 0.2 ml. To facilitate later observation of fibroblast cells, some aliquots were planted into 5 ml plastic Cooper tissue culture dishes[2]. The remainder were planted into 15 ml capped culture tubes for development of erythroid and myeloid cells. All aliquots, and the sample containing Velban, were placed into a 37°C incubator as described before. After 24 and 48 hours, Vel-

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[1]Velban. Eli Lilly and Co., Indianapolis, Indiana.

[2]Cooper tissue culture dishes. Falcon Plastics, Los Angeles, Calif.

ban was added to some aliquots to effect mitotic arrest.

Fibroblast-like cells were found attached to the Cooper dishes after vigorously flushing away the loose marrow cells with a jet of warmed medium 24 hours after planting. Direct observation with phase-contrast microscopy revealed several hundred cells attached to each dish. Twenty-four and 48 hours after medium replenishment and continued incubation, Velban was added to the dishes to effect mitotic arrest.

Chromosome preparations were made from the cultured cells using minor modifications of standard methods for lymphocytes [50,59,75] and marrow [62, 132]. Three hours after addition of Velban, tube contents were mixed vigorously and transferred to unsterile conical 15 ml centrifuge tubes. The cells were washed once in physiological saline solution by centrifugation at 200 gravities for 5 minutes. Approximately 0.1-0.2 ml of loosely packed cells were suspended by vigorous agitation in 4 ml of 0.075 M KCl [59]. After 5 minutes (lymphocytes) or 10 minutes (marrow) the cell suspension was centrifuged for 5 minutes at 200 gravities. The supernatant fluid was discarded, leaving approximately 0.2 ml in which the cells were thoroughly resuspended. One ml of fixative (glacial acetic acid 3 parts, with methanol 1 part, by volume) was added slowly to the suspension. The cells were

allowed to remain undisturbed at room temperature for 15 minutes.

Squash chromosome preparations were made by carefully pipetting a small volume of cells from the bottom of the centrifuge tube, and placing them into about 0.2 ml of lactic-propiono-orcein stain (LPO)[1] [30]. A small drop of the vigorously mixed stain/cell suspension was placed under a cover-slip on a clean glass slide. The cells were squashed by firm pressure on the cover-slip under layers of filter paper, and the cover-slip edges were sealed with paraffin or rubber cement.

Air-dried preparations [104] were made by washing the fixed cells in three changes of fixative, leaving the final suspension lightly hazy. Very small drops of the final suspension were placed on very clean glass slides and quickly dried with an electric hair dryer. These preparations were stained for 20 minutes in LPO, or 5 minutes in 3% giemsa stain[2] at pH 6.0. After a brief rinse in distilled water, slides were dried and Permount[3] was used to attach a #1 1/2 thinness cover-slip.

Chromosome preparations of fibroblast-like cells

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[1]Orcein, natural. G.T. Gurr, London, England.

[2]Uni-Tech Chemical Manufacturing Co., Sun Valley, Cal.

[3]Permount. Fisher Scientific Co., Fair Lawn, N. J.

attached to Cooper dishes were made in situ. At 3, 8, or 24 hours after the addition of Velban, the medium was carefully removed by aspiration. Hypotonic KCl was added to half-fill the dish, and after 15 minutes 1 ml of fixative was added slowly. After remaining undisturbed at room temperature for 15 minutes, the fluid was half-removed by aspiration and a similar volume of fixative was added slowly. This process was repeated once, all fluid was removed, and undiluted fixative was added slowly to fill the dish. After 10 minutes the fixative was removed by aspiration, the bottom of the dish was dried quickly with an electric hair dryer, followed by staining in situ with LPO or giemsa. After rinsing and drying, the walls of the dish were removed, and temporary mounts were made with glycerin and glass cover-slips.

Both observation of chromosomes and photomicrography were performed with Zeiss microscopes and attachment cameras, and later with the Zeiss Photomicroscope II. Critical observations were made with an achromatic-aplanatic N.A. 1.4 condensor oiled to the slide and a Planapochromatic 100x N.A. 1.3 oil-immersion objective. Visual observation was performed at 1250 diameters magnification; photographs were taken at 400 diameters and later enlarged to 3000 diameters magnification. Good contrast and resolution were obtained through the use of a 550 millimicron wavelength inter-

ference filter in the illumination path.

Karyotypes were made from a number of photographed metaphase cells. Negatives on Kodak High Contrast Copy film were developed in D-41[1] to retain good tonal rendition. Prints were made on Kodak Kodabromide or Dupont Varigam enlarging paper, usually contrast grade 4. Chromosomes cut from prints were arranged on white cardboard in 7 groups, plus the sex chromosomes, according to the agreements of the Chicago Conference [22]; chromosomes 1-3, 16-18, and Y were individually identified. When possible, abnormal chromosomes were identified according to the chromosomes from which they were derived.

Chromosome aberration scoring was performed after selection of well-spread metaphase cells under low power; selected cells were examined at 1500-2000 diameters magnification. Seventeen distinct classes of chromosome aberration were tabulated to retain the information content of the visual observations; very rare aberrations were described separately whenever encountered. Figure 4 is a list of the tabulated aberrations, with symbolic representations of their metaphase appearance.

Estimates of the expected frequencies of pair-

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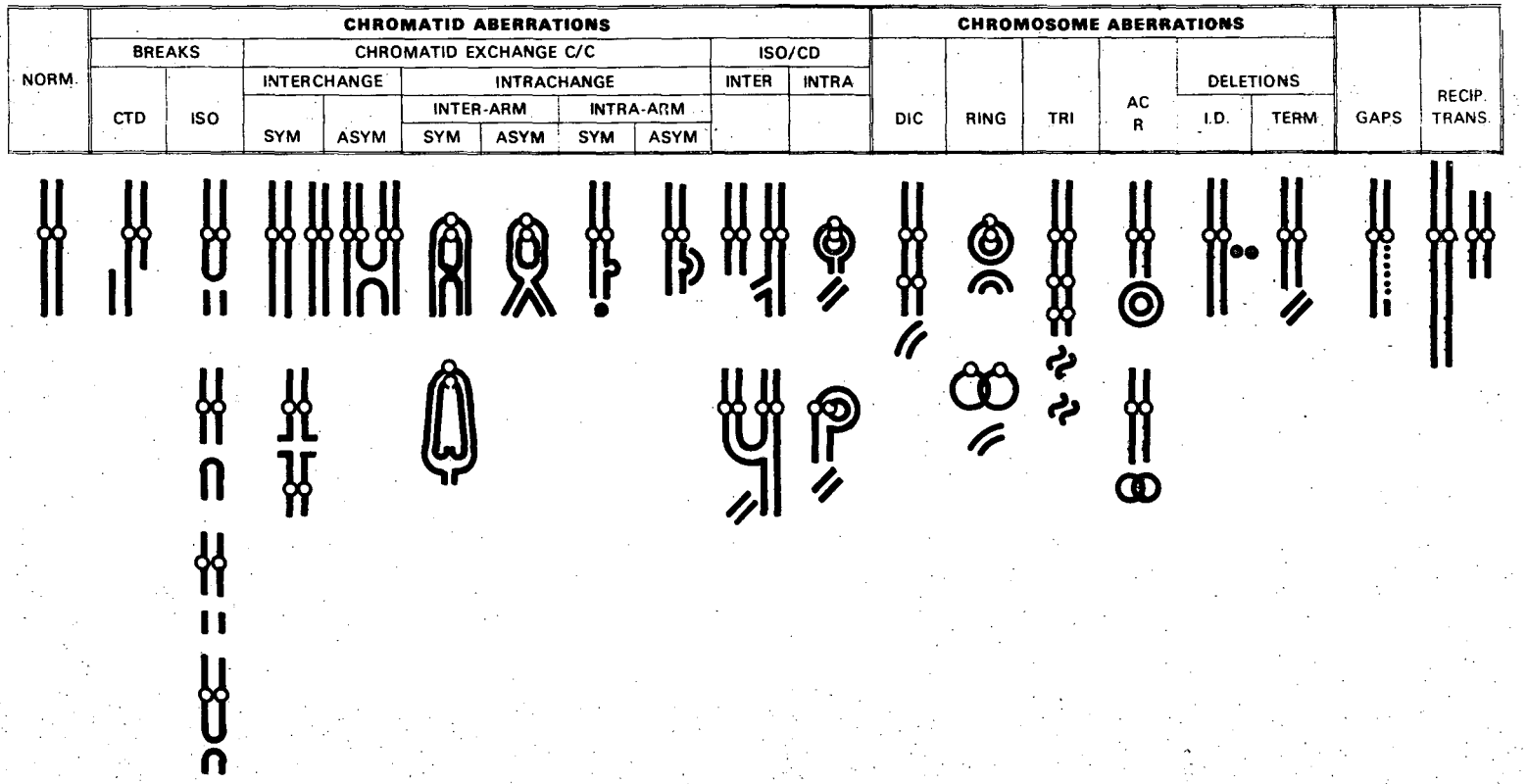
[1]D-41 is made by the addition of 0.12 grams benzotriazole (Kodak Anti-Fog #1) to each gallon of Kodak Developer D-76. Negatives are developed according to the schedule for D-76.

Figure 4. Types of chromosome aberrations scored during the study.

Column headings are terminology based on the assumption that chromosomes or chromatids break either during G_1 or G_2+S of the cell cycle, followed by reunion of the broken ends. Diagramatic chromosome aberrations represent the configurations as seen at metaphase; the locations of the putative breaks are not indicated.


NORM.
CHROMATID ABERRATIONS
BREAKS
CTD
ISO
CHROMATID EXCHANGE C/C
INTERCHANGE
SYM
ASYM
INTRACHANGE
INTER-ARM
SYM
ASYM
INTRA-ARM
SYM
ASYM
ISO/CD
INTER
INTRA
CHROMOSOME ABERRATIONS
DIC
RING
TRI
AC R
DELETIONS
I.D.
TERM
GAPS
RECIP. TRANS.

DBL 734-5128

wise chromosome combinations in chromatid exchanges were obtained in the following way: The chromosome lengths given by the Chicago Conference report [22] were recomputed to give chromosome lengths as percentages of the complete female haploid set. The total length of each chromosome group as a fraction of the haploid set length was taken as proportional to the probability that a member of the group would be involved in an exchange. The product of the probabilities of two groups was taken as the probability of interaction between members of the groups to form chromatid exchanges. The probabilities of like and indistinguishable pairwise combinations were summed. The resultant figures were taken as the expected frequencies in chromatid exchanges of the different pairwise combinations, and they are tabulated in Table 3 along with the observed frequencies. This very simple model assumes that the expected interaction by classes will be some random function of both chromosome length and numbers in each class.

Results

Bone marrow cells and marrow-derived fibroblast-like cells were obtained first from the propositus in late October 1967. Patty's lymphocytes were cultured first in early November 1967. The distribution of chromosomes per cell was determined from 50 or more

cells from each of marrow and lymphocyte cultures; the results are given in Table 2. The chromosome abberation frequency in both marrow and cultured lymphocytes was obtained from observation of 200 cells in each sample; the results are shown in Table 3. The frequencies with which different chromosome classes were represented in chromatid exchanges, found by observation of 100 cells, are shown in Table 4. A representative marrow cell karyotype is shown in Figure 5, while a commonly seen abnormal interphase marrow cell is shown in Figure 6. A karyotype obtained from lymphocyte culture, containing an A/D chromatid exchange, is shown in Figure 7, and additional chromatid exchanges are shown in Figure 8.

In these initial cultures of both lymphocytes and bone marrow cells from the propositus, the distribution of numbers of chromosomes per cell is that expected of normal humans[1]. There is a sharp mode at 46 chromosomes per cell; the straggling incidence of lower numbers likely is a preparative artifact. Cell lines with hyperdiploid chromosome numbers are not seen; specifically there is no tetraploidy or endoreduplication.

The chromosome aberration frequency seen in bone marrow cells, directly after biopsy and also after 24

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[1]Loughman, W.D. Unpublished observations of over 8000 cells from 14 clinically normal individuals.

Table 2. Distribution of number of chromosomes per cell in marrow and cultured lymphocytes of the FA propositus

	Number of chromosomes per cell											
	≤ 40	41	42	43	44	45	46	47	≥ 48	~4n	>4n	Total cells
Marrow	6[1]	2	4		2	6	78			2		100
Lymphocyte		0.7		1.3	2.7	3.3	92.0					150
Marrow fibroblast	No dividing cells found											500

[1]Tabulated values are percent of cells observed.

Table 3. Initial frequencies of chromosome aberrations in marrow and lymphocytes from the FA propositus.

	CHROMATID ABERRATIONS										CHROMOSOME ABERRATIONS						
	BREAKS		CHROMATID EXCHANGE C/C						ISO/CD								
			INTERCHANGE		INTRACHANGE				INTER	INTRA					DELETIONS		
					INTER-ARM		INTRA-ARM										
	CTD	ISO	SYM	ASYM	SYM	ASYM	SYM	ASYM			DIC	RING	TRI	ACR	I.D.	TERM	GAPS
MARROW																	
3 hour	0.5																2.5
24 hour	0.5										0.5						1.5
48 hour	1.5															0.5	2.5
LYMPHOCYTES	5.5	1.0	5.0	4.5		0.5			2.5		4.5						9.0

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200 cells were observed in each sample; tabulated values are frequencies per 100 cells = aberration percentage.

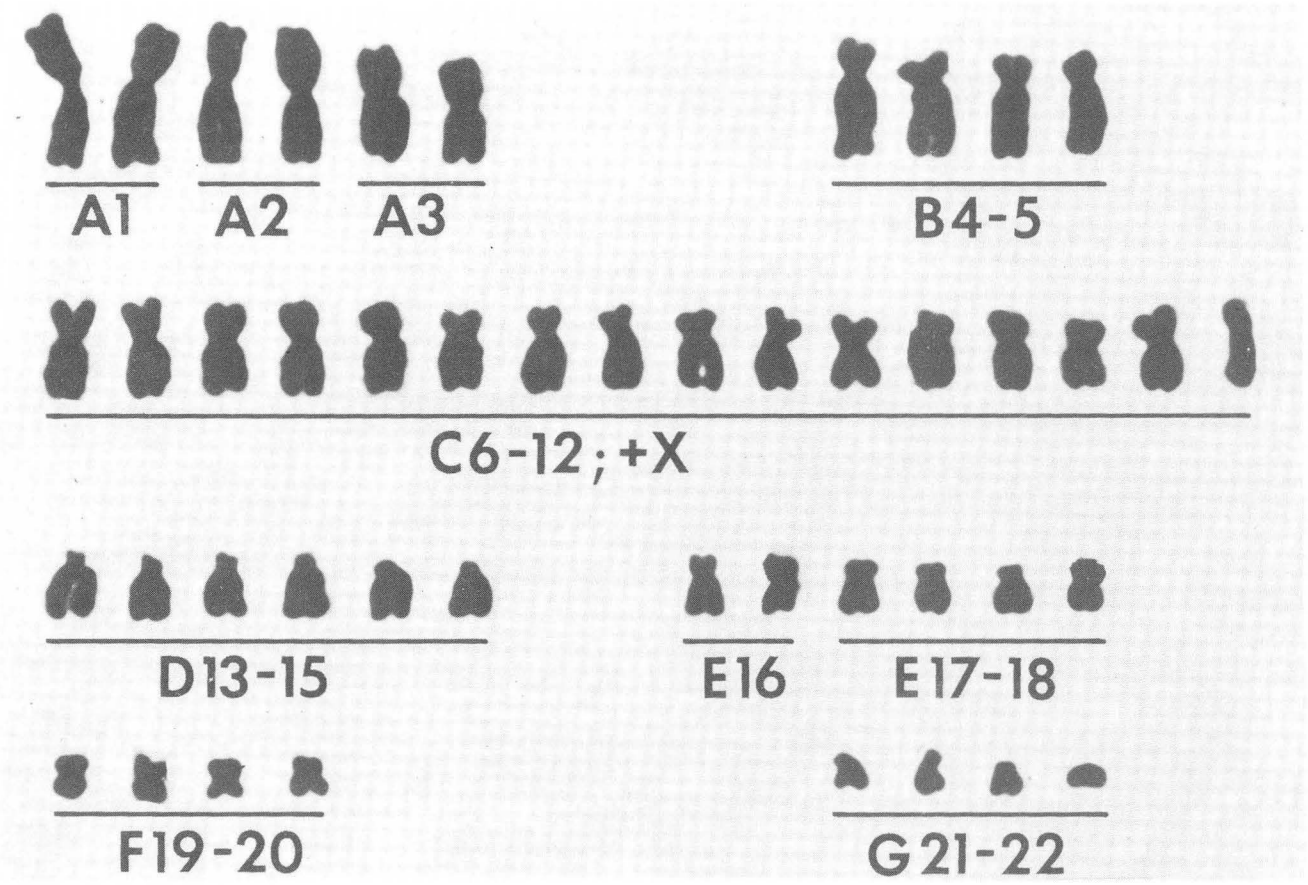
Table 4. Chromosome associations among chromatid interchanges in lymphocytes of the FA propositus (Nov. 1967).

	A	B	C + X	D	E	F	G
A (23.24)	**11** *5.40*						
B (11.98)	**8** *5.74*	**3** *1.44*					
C + X (38.29)	**21** *17.80*	**9** *9.18*	**22** *14.66*				
D (9.98)	**9** *4.64*	**2** *2.4*	**4** *7.64*	**None** *1.0*			
E (8.63)	**2** *4.02*	**1** *2.06*	**3** *6.6*	**1** *1.72*	**None** *0.74*		
F (4.58)	**1** *2.12*	**None** *1.1*	**1** *3.5*	**None** *0.92*	**None** *0.8*	**None** *0.21*	
G (3.52)	**1** *1.54*	**None** *0.8*	**1** *2.54*	**None** *0.66*	**None** *0.58*	**None** *0.3*	**None** *0.11*

DBL 734-5111

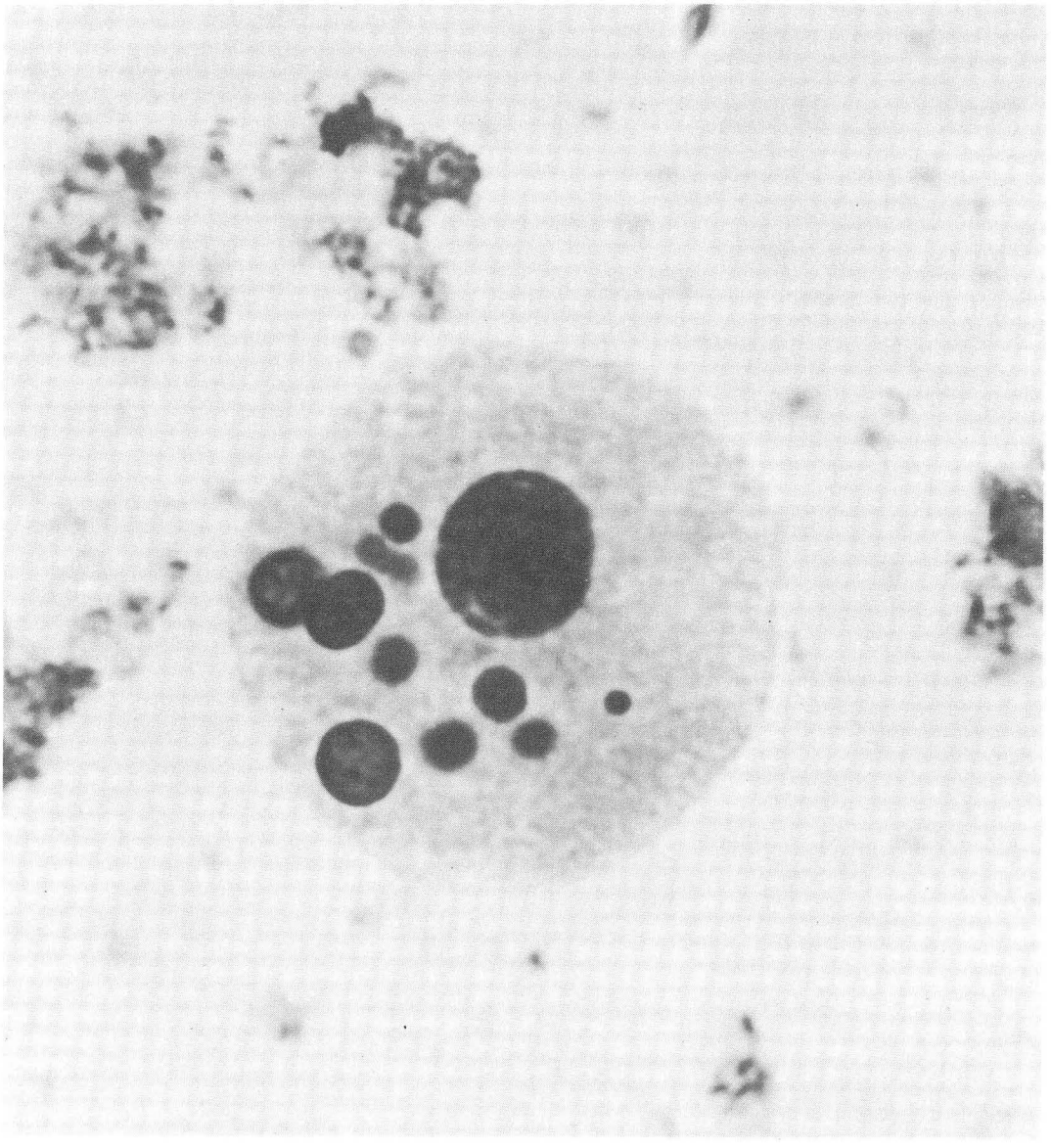
100 cells were examined; block numbers are observed frequencies, italic numbers are expected frequencies (see text). Numbers in parentheses are summed lengths of chromosomes in each class expressed as percentages of the female haploid set length.

Figure 5. Representative normal karyotype from a marrow cell of the FA propositus (Oct. 1967). Magnification = 3000x.



XBB 734-2455

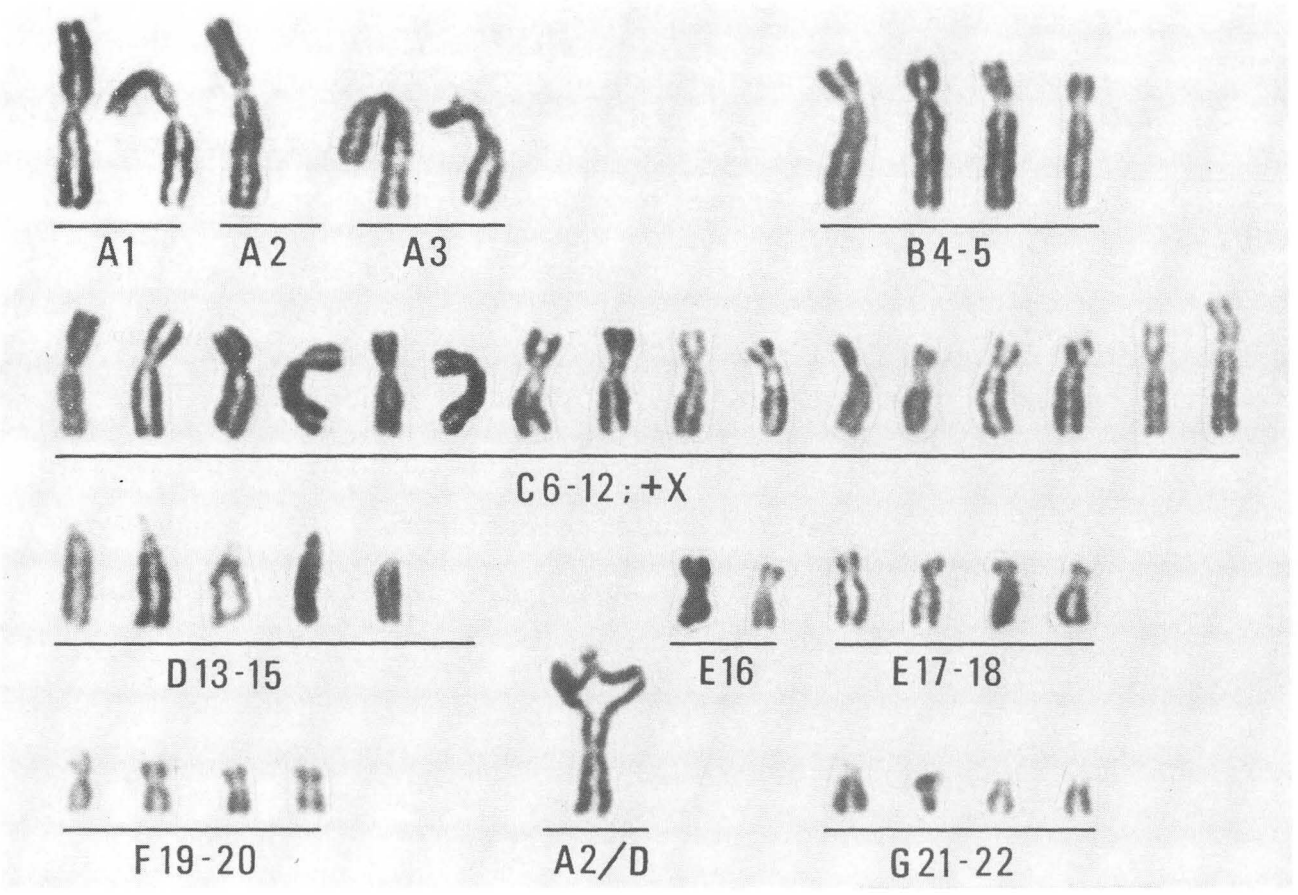
Figure 6. Representative abnormal interphase cell from the marrow of the FA propositus (Oct. 1967). Most such cells had similar numbers of micronuclei. A few had only one or two, while rarely extreme nuclear fragmentation and destruction was found. Anaphase configurations were rather uncommon, but no instances of anaphase bridging were found. Magnification = 3000x.



XBB 734-2453

Figure 7. Representative abnormal karyotype from a cultured lymphocyte of the FA propositus (Nov. 1967).

This cell contains no chromatid breaks or other aberration besides the symmetrical chromatid interchange shown. The interchange is between members of the A and D groups, clearly showing the participation in exchanges of non-homologous chromosomes. It is the long arms of the chromosomes which are involved; this was a common occurrence (see text). Magnification = 3000x.



XBB 734-2454

Figure 8. Chromatid aberrations from cultured lymphocytes of the FA propositus.

These chromosomes from different cells have been identified by karyotyping in November 1967. Size variation among homologous chromosomes are due to technical differences; magnification = 3000x.

a,c-f: symmetrical chromatid interchanges.

b, g-k: asymmetrical chromatid interchanges; h is incomplete.

l-n: isochromatid/chromatid interchanges with two centromeres, found in cells without visible fragments.

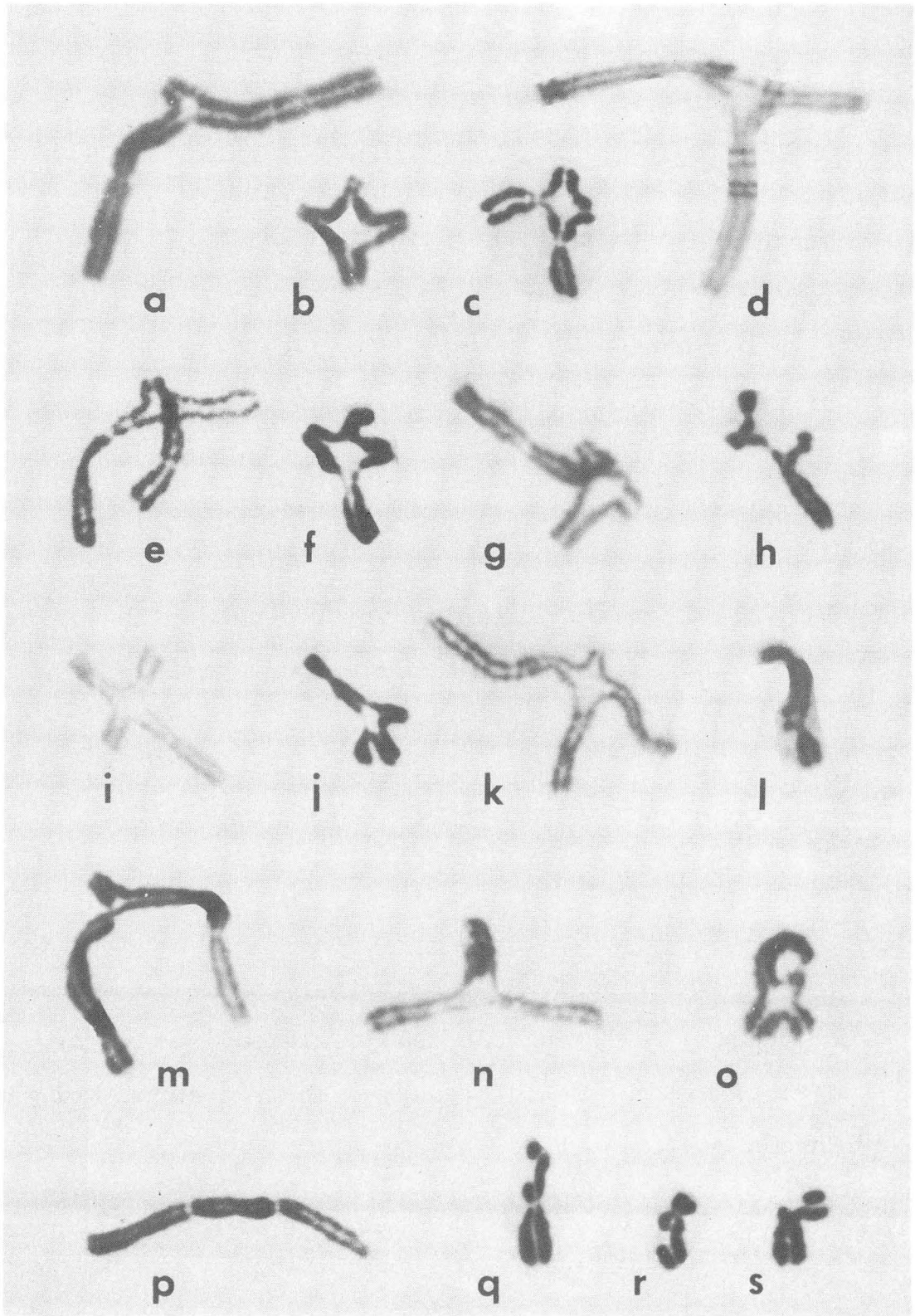
o: asymmetrical inter-arm intrachange; the only intrachange in 2100 cells from 7 cultures.

p: dicentric found in a complete cell without visible fragments. Most such aberrations are considered here to arise from asymmetrical chromatid interchanges following one cell division (see text).

q-s: simple chromatid breaks; s shows a chromatid gap.

Chromosome identities:

a = A1/A1; b = D/D; c = A3/A3; d = A1/B; e = A/C; f = C/E; g = A/D; h = C/E; i = A/C; j = C/E; k = A/B; l = C/E; m = A1/A1; n = C/C; o = A2; p = A/A; q-s = C-group.



XBB 734-2450

and 48 hours of culture, is both unremarkable and quite normal. This is an unexpected finding, for almost 3% of the interphase cells in the patient's marrow biopsy had the appearance of the cell in Figure 6. Such multiple micronucleated cells are often seen in cultured cells after X-ray or radiomimetic drug exposure, and they are associated then with extensive chromosome damage.

The chromosome aberration frequency found in cultured lymphocytes is striking: Simple chromatid breaks, and also dicentric chromosomes, are elevated 5- to 10-fold above normal values. The 12% incidence of chromatid interchanges is 700 times normal[1] [70], and twice the incidence of simple breaks ($t = 2.05$; $P < 0.05$). All other aberration categories, including other forms of chromatid exchange, are virtually nonexistent.

Table 4 shows that non-homologous chromosomes as well as homologs participate in chromatid exchanges. The longer chromosomes, homologous and non-homologous alike, are more frequently involved than the shorter chromosomes.

Fibroblast cultures exhibited no perceptible growth, and no dividing cells were obtained even after

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[1]Loughman, W.D. Unpublished data. One chromatid interchange in 8000 cells of normal individuals = 0.0125%. Combining this and the data given in [70] gives a frequency of 0.017% (2/11,720).

extended exposure to Velban. At the second day of culture the cells were firmly attached to the culture dish, well isolated one from another, and without evidence of colony formation. Marked cells were observed by phase microscopy at intervals for seven days. Neither cell division nor colonies could be found. The cells had normal-appearing intracellular features. Fully 98% of the cells excluded eosin[1] dye within the half-hour after its introduction into the cultures on the fifth and seventh days. By the criteria of dye exclusion, normal appearance under phase microscopy, and continued attachment to the culture dish as single cells, these cells remained both viable and non-dividing for seven days after biopsy.

Attempts to grow marrow-derived fibroblasts from this FA patient were made on three additional occasions in the following three years. None of the cultures were ever vigorous and the division rate was always far too low to be useful; the attempts were abandoned.

Discussion

The failure to grow fibroblasts from marrow biopsies was disappointing; a study of these cells con-

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[1]Eosin B, Allied Chemical and Dye Corp., National Aniline Division, New York, N.Y. Stock solution 0.01% w/v in physiological saline, 10 drops from #21 gauge hypodermic needle for each 5 cc. of culture medium.

current with those on lymphocytes was desirable. Yet the failure itself was interesting; it suggested a possible metabolic or nutritional deficiency. A simple experiment to check this possibility is described in a later section.

Even more interesting was the unusually high frequency of micronucleated cells, without any evidence of serious chromosome damage, in the patient's marrow. This suggested two cell populations, one subject to the lethally damaging effects of some agent, and the other escaping its effects. Since others have claimed that a viral agent might be important in the etiology of FA, it was supposed that a past viral infection could have affected many cells then present in the marrow. The cells observed in this study would then represent both the remaining lethally affected cells, and the normal cell population from the survivors which repopulated the marrow. This possibility seemed a bit strained, but with other observations described later it prompted a search for virus by electron microscopy. The results are presented in a later section.

The general observation that this patient has a normal karyotype, without any constant and specific numerical or structural chromosome abnormality, is in accord with all previous reports. The absence of endoreduplication contrasts with the findings of others

[11,55,91,115,117], but agrees with that of Schmid [108].

Every previous cytogenetic study of FA has revealed a marked elevation in non-specific structural abnormalities of chromosomes in cultured blood cells. The present study is no exception. Some workers have found these aberrations in marrow cells as well [128]; the present work reveals none in this patient's marrow, confirming the observations of both Bloom et al. [11] and Schmid [109].

German claimed that chromatid exchanges in FA lymphocytes represented somatic crossing-over [39,43]. Crossing-over is thought of usually as being restricted to homologous chromosomes. In this sense, the data presented here, like that given by Obe [85], cannot support the contention. From Table 4 it is found that homologs are more frequently found in exchanges than might be expected (36% found; 23.6% expected), and from Table 3 it is found that intrachanges are rare. If the concept of crossing-over is modified to include exchange between non-homologous chromosomes, the relative lack of intrachanges could support German's view.

The increased frequency of homologs in exchanges is found entirely among the longer chromosomes. The increased involvement of longer chromosomes is general, and not restricted to homologs. Non-homologous ex-

changes in groups B and C are expected in 32.7% of exchanges, but found in 38%. If all long chromosomes are considered (groups A through C), 54.2% are expected, but 74% are observed. Conversely, the short chromosomes (groups D through F) are expected to be involved in 45.8% of exchanges but are found in only 26%.

The observations accord with expectation of exchange association as a function both of chromosome length and of numbers of chromosomes with similar lengths. However the expectation of association frequencies simply proportionate to the product of chromosome lengths must be modified. Short chromosomes are found to be less frequently involved than expected and longer ones more frequently, thus confirming a previous observation on induced exchanges [85]. Thus the probability of a chromosome's involvement in chromatid exchanges may increase exponentially with an increase in its length. Elkind and Whitmore have discussed this possibility in a different context, and agree on its likelihood [31, p. 455]. Griffin et al. have found induced breakage in Bufo chromosomes is exponentially related to chromosome length [45].

A very large body of evidence supports the present-day view that chromosome rearrangements are the result of chromosome breakage followed by incorrect reunion of the broken ends [31]. In this "breakage

first" model, the incidence of rearrangements involving one break in each of two chromosomes is a function of the product of the separate frequencies of simple breaks. To a rough approximation, the five- to ten-fold elevation of simple chromatid breaks observed should be associated with a 25- to 100-fold increase in chromatid exchanges. Yet the 12% observed incidence is roughly an order-of-magnitude higher than would be predicted by this model. Further, the model would predict an elevation in other aberrations as well. Apart from a modest increase in the frequency of dicentric chromosomes, this is not seen.

In short then, in this patient's cells the ratio of simple breaks to exchanges is inverted, exchanges are more frequent than expected, and dicentrics are virtually the only other aberration class seen in this FA patient's cells.

Among other sources, dicentric chromosomes can arise from chromatid exchanges after one cell division [e.g. 118]. It is generally agreed that many of the dividing cells in 68-hour cultures of human blood are in the second or even later division. Thus the dicentric chromosomes seen in the patient's lymphocyte cultures may be derivatives of chromatid interchanges. The data in Table 2 support this view: Chromosome-type breaks, the other likely precursors of dicentric chromosomes, are absent along with virtually every

other chromosome-type aberration.

Having raised the possibility that only chromatid aberrations need be considered in this patient's cells, it may be argued that the damaging mechanism operates only when the chromosomes are functionally double, in the S and G_2 stages of the cell cycle. While this cannot be excluded as a possibility, it is not so easy to imagine a chromosome breakage mechanism which is both restricted to a small portion of the cell cycle, and also restricted as to the specific type of abnormality arising from misrepair of the breaks.

If the "breakage first" hypothesis is an unsatisfying model for rationalizing the pattern of chromosome aberrations, another model must be sought. A plausible alternative to the "breakage first" model has been proposed by Revell [99,100]. In his "incomplete exchange" model, most simple breaks and their presumed derivatives are the consequences of incomplete exchanges of chromosome parts. This model does not permit explanation of the virtual absence in the FA case of chromosome aberrations other than the three discussed above. However even more difficult questions are raised if the "breakage first" model is applied to the data. Brewen and Brock [14], Heddle and Bodycote [51], and Heddle et al. [53] discussed the alternatives and conclude that certain aspects of the exchange model could be correct in part. Chromosome aberra-

tions in normal cells therefore may arise from two independent and co-existent mechanisms.

A Working Hypothesis

As a working hypothesis then, it may be argued that a normally occurring exchange process is both predominant and also aberrant in the FA case's lymphocytes. Direct tests of this hypothesis are not made readily in the material at hand. The alternative, predominance of the breakage-and-reunion process, may be tested by examining its consequences in the patient's cells. Through exclusion, failure to verify predictions from the breakage model would lend support to the exchange hypothesis.

On the "breakage first" hypothesis, explanation of the inverted ratio of chromatid breaks to chromatid interchanges clearly must require a greatly augmented reunion process: A greatly increased frequency of chromatid breaks would be masked by this augmented repair, as many more breaks than usual would "heal" and become unobservable. At the same time, a proportionate increase in mis-repair could be expected. The high frequency of chromatid exchanges would be the observable evidence of this. With both increased breakage and increased repair and mis-repair, the inverted ratio could be obtained.

Augmentation of a normal chromosome repair process

could occur in at least two ways: There could be a longer-than-normal time available for chromatid break repair at the normal rate (lengthened S or G_2 cell cycle stages); or the rate of chromosome repair could be higher than normal. The time available for repair may be determined by direct analysis of the time segments of the cell cycle. Should "unscheduled" DNA synthesis occur [88], it could be observed incidental to this analysis. The rate of chromosome repair may be inferred by observing directly the time required for repair of X-ray induced chromosome breaks [142].

An alternative, and opposite, explanation for the inverted ratio of exchanges to breaks is given by Schroeder [112,113]. She has proposed that some FA patients are deficient in hexokinase, the rate limiting enzyme in the glycolytic metabolic pathway. Reduced glycolysis would lead to a reduction in the levels of cellular ATP needed for normal chromosome repair. "Inefficient" repair would lead to both unrepaired breaks and mis-repaired breaks. A deficiency of hexokinase could explain the poor growth of fibroblasts from this FA patient, but could not explain fully the data given in Table 3. Hexokinase may be determined by direct biochemical analysis.

The "breakage first" hypothesis requires an increase in simple breaks before an increase in complex rearrangements may be observed. This is tantamount to

requiring the "increased fragility" or "instability" of FA chromosomes which has been postulated [35,41, 116,128]. Evidence purporting to demonstrate this fragility has been presented [54,117], but these investigators had not ruled out alternative explanations. The "exchange first" hypothesis does not require chromosome susceptibility to simple breaks, and failure to find the postulated increased susceptibility to induced breakage would lend support to the hypothesis. Comparison of X-ray induced chromosome breakage rates in FA and control cells might be made in the same experiment used to determine chromosome repair times. The susceptibility to breakage of chromosomes during the S and G_2 stages of the cell cycle might be assessed by measuring induced aberration frequencies following X-irradiation during those periods.

Apart from the hypotheses just described, the corollaries of the increased chromosome aberration frequency in FA are of considerable interest, and a number of questions may be asked. Do the chromosome aberrations lead to decreased survival of the cells containing them? Many circulating lymphocytes are long-lived cells [83]: Are the aberrations the result of damage accumulated during the long interphase, or do they arise <u>de novo</u> in each cell generation? Are any of the cytogenetic abnormalities found in the patient's

parents, presumably heterozygous for the FA gene? Previous reports of cytogenetic investigations in FA agree on many points, yet there are also areas of great disagreement: Is the frequency of aberrations found in FA cells constant through the course of the disease? Is the pattern of aberrations constant through the course of the disease? Does the medical treatment influence the aberration frequency or pattern?

The following sections describe the results of experiments designed to test the hypotheses given, and the questions asked above. A number of additional questions were raised by the sometimes unexpected results; these too are answered by experiment whenever possible.

VIABILITY AND MITOTIC INDEX OF CULTURED LYMPHOCYTES

A number of trial experiments with the patient's lymphocytes were failures. On grounds of abnormal cell morphology and staining reactions, some cultures were subject to a very high cell death rate, while others appeared to be much less affected. Accordingly, a study of the *in vitro* viability of the patient's lymphocytes was made.

Some of the trial experiments, e.g. those on chromosome repair time, required cells in the first division after experimental treatment. In some of these cultures no dividing cells could be found until the third day. This contrasted with experience showing dividing cells in normal cultures as early as 30 hours after exposure to PHA-M. The subjective impression gained was of great variability in mitotic index patterns between cultures established on different dates. Therefore the time-dependent variation of the mitotic index in the FA patient's cultured lymphocytes was studied.

Materials and Methods

The *in vitro* mitotic index pattern of the patient's lymphocytes was assessed as follows: Lymphocyte cultures were established and incubated as previously described, with antibiotics added to some cultures but omitted in others. At 24 hours of culture, and at 5-

hour intervals thereafter to a total of 90 hours, 0.2 ml of medium and cells from a well-mixed culture were pipetted into a centrifuge tube. After centrifugation for 5 minutes at 1000 g, the supernatant medium was discarded and a small drop of LPO stain was added. Following thorough mixing of the stain and cells by repeated pipetting with a Pasteur pipette, a small drop of cell suspension was placed on a microscope slide under a coverslip. After squashing the preparation under blotting paper, it was examined under the microscope using a 25X dry objective at a final magnification of 310 diameters. The microscope ocular was fitted with a reticulocyte counting reticle, and a differential count of 4000 cells was made by scoring cells in interphase and cells in any stage of mitosis. The equivocal status of occasional cells was resolved by examination with a 100X oil-immersion objective at a final magnification of 1600 diameters. To avoid removal of excessive medium and cells from any one culture, several replicate cultures were used for alternate sampling. Interculture variation was controlled by occasional interculture cross-checking of results. These procedures were followed both for cultures with antibiotics, and for those without. When there were no significant differences between cultures with and without antiobiotics, as determined by standard statistical procedures ("Student's" t test [123,125]), these

results were combined. Standard statistical methods were used to place 95% confidence limits on the determinations [25]; all statistical computations were carried out on a small computer[1]. Results were expressed as percent of cells in division, and plotted as a function of time after culture initiation. All the above procedures were repeated on cells from a normal (control) individual.

The in vitro viability of the patient's lymphocytes was assessed as follows: Lymphocyte cultures were established as previously described. Some cultures were provided with PHA, some with antibiotics only, and some with both. At the time of culture initiation, and at 24-hour intervals thereafter to a total of 168 hours, 0.2 ml of medium and cells from well-mixed cultures were pipetted into small vessels containing a volumetrically negligible quantity of dry, powdered eosin B. After thorough mixing a portion of the stained cell suspension was placed into a hemacytometer which was stored in a small moist chamber to allow the cells to settle. After 10 minutes the cells were examined and counted under the microscope at a magnification of 400 diameters. A differential count of pink-stained (dead) and viable (colorless) cells was made, along with a total cell count.

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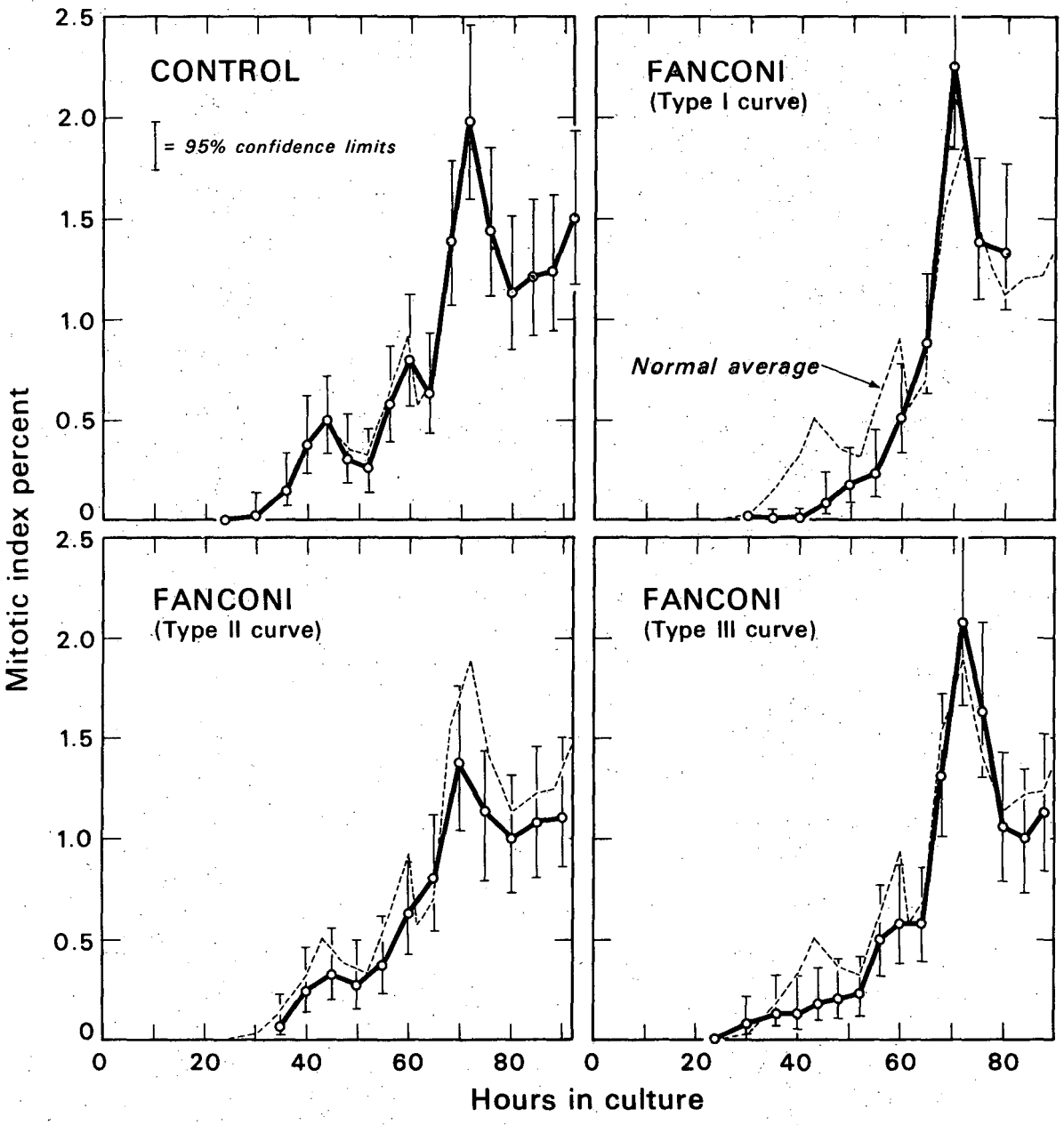
[1]Programma 101. Olivetti-Underwood Corporation

The total cell count was normalized to total cells in 10 ml of culture through multiplication by factors accounting for cell loss by sampling and for dilution during counting. Total viable cells was computed by multiplying the percent viable cells by the total cells in 10 ml. These procedures were performed on cultures with and without antibiotics, and when no significant differences were found the resulting data were combined. The results were expressed as percent viable cells, and as total cells and total viable cells as percentages of the original inoculum. All were plotted as functions of time after culture initiation. As before, data differences were assessed for significance and 95% confidence limits were computed using standard statistical methods [25,123,125]. All procedures were repeated on the cells of a normal (control) individual.

Results

The results of the mitotic index determinations are shown in Figures 9 and 10. Five separate determinations were made on the propositus's cells, and two on control cells, in March, June, August, and September of 1969. Dividing cells could be found at low frequency in control cultures as early as 30 hours after culture start. The graph showing the frequency of dividing cells is a curve which rises steadily with

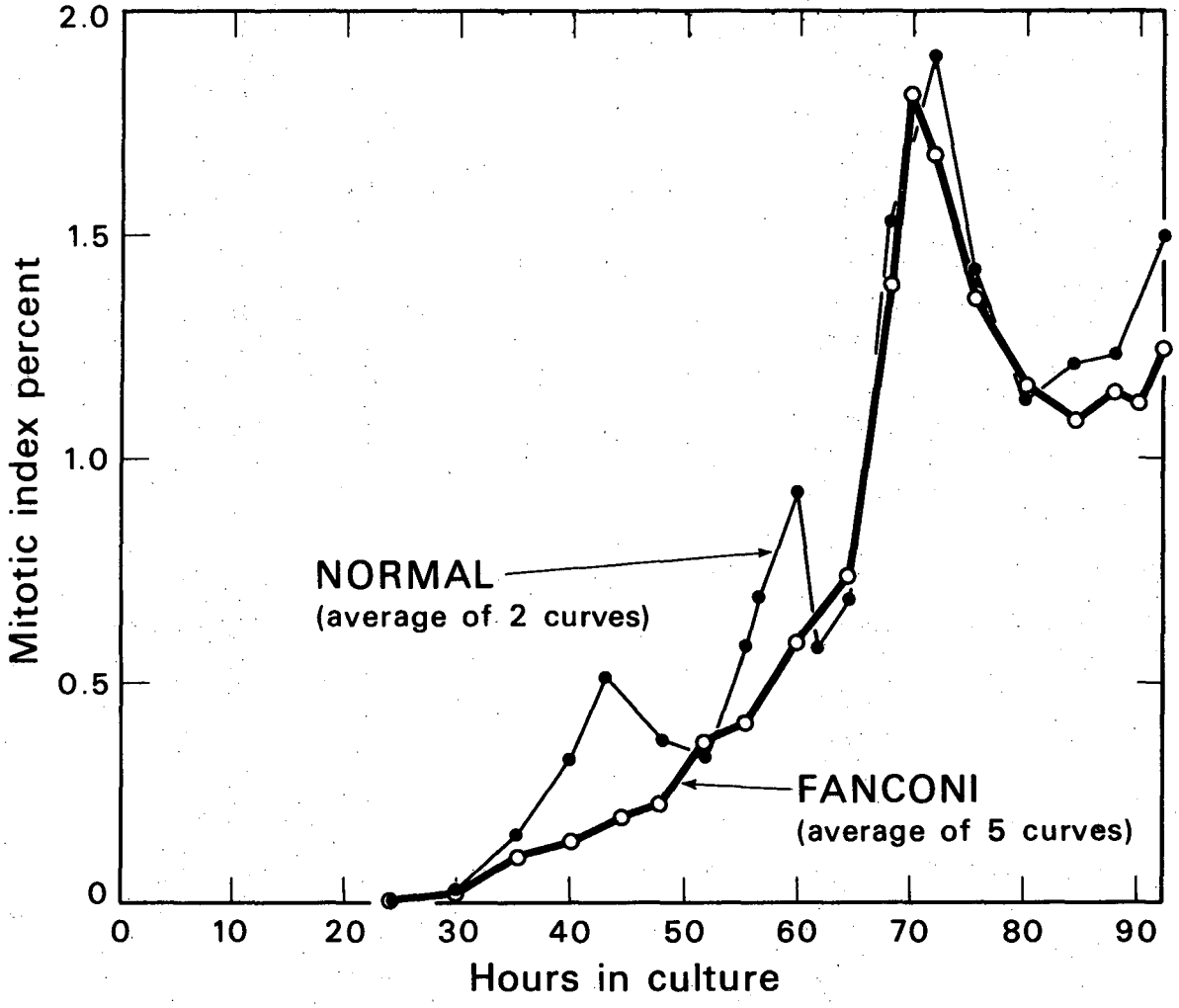
Figure 9. Mitotic index patterns of cultured lymphocytes: FA cells compared with normal cells. The dotted line in all four graphs is the average mitotic index pattern of two different control cultures. In the FA curves, either the first mitotic peak at 44 hours is reduced (Type III curve), or the second at 60 hours is reduced (Type II curve), or both early peaks are reduced (Type I curve). Each type of FA curve has been found on at least two occasions. Notice that although the early peaks are reduced in amplitude, dividing cells are present in the cultures. The third peak in all FA cultures appears to be of normal amplitude and time of occurrence.


CONTROL
= 95% confidence limits
FANCONI
(Type I curve)
Normal average
FANCONI
(Type II curve)
FANCONI
(Type III curve)
Mitotic index percent
Hours in culture
0
0.5
1.0
1.5
2.0
2.5
0
20
40
60
80

DBL 704 5675

Figure 10. The average mitotic index pattern of cultured FA lymphocytes.

The average mitotic index pattern of five FA lymphocyte cultures is compared with the average mitotic index pattern of two normal control cultures. The absence of the first two mitotic peaks in the FA composite, with a mitotic index steadily rising with time, suggests considerable division asynchrony among cells of a normally synchronous lymphocyte subpopulation.


2.0
1.5
1.0
0.5
0
Mitotic index percent
NORMAL
(average of 2 curves)
FANCONI
(average of 5 curves)
0
10
20
30
40
50
60
70
80
90
Hours in culture

DBL 704 5672

time, and which has distinct peaks at 44, 60, and 72 hours, with the suggestion of another peak occurring later than 92 hours.

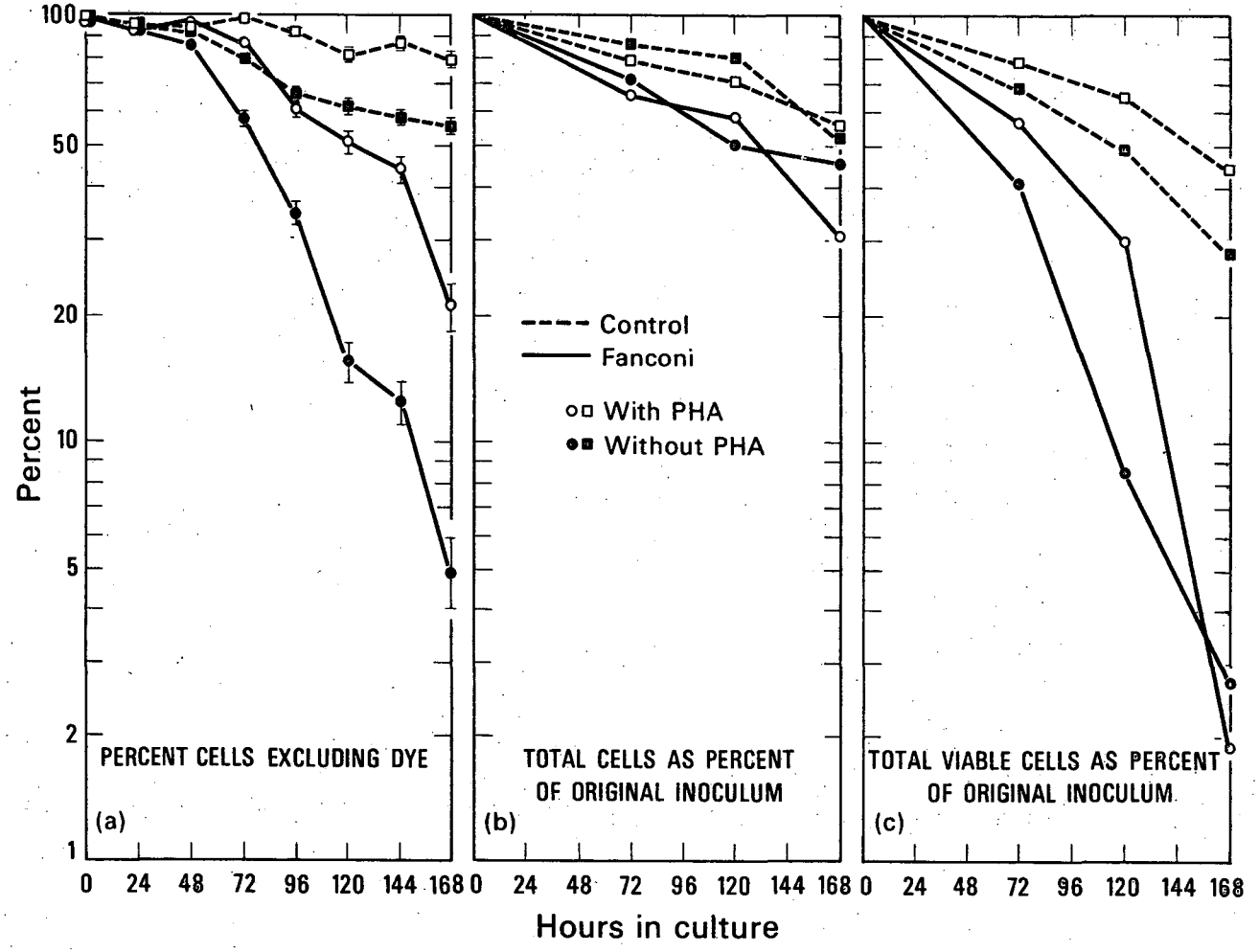
Surprisingly, mitotic index curves from the FA cultures are of three types: In the first, both early peaks are absent or greatly reduced. In the second type the first peak is present but the second is absent; the reverse is true in the third type of curve. All three curves show a peak of mitotic activity which corresponds well to the third peak in control curves. There was no difference between cultures treated with antibiotics and those without antibiotics. There was no apparent pattern to the sequence of these curves, and no apparent relationship with clinical data or treatment modes.

The results of the in vitro viability studies are shown in Figure 11. The graphs pertain to a study made in February 1970, but less detailed determinations in 1969 and 1971 agree well with them. There were no significant differences between cultures with and without antibiotics, and the data were combined. All cultures show reduced viability with time, but those without PHA showed the greatest reduction. Cells from the propositus are markedly less viable than those from controls.

In control cultures without PHA, the percent viable cells declines exponentially with a half-time

Figure 11. Survival *in vitro* of cultured lymphocytes: FA cells compared with normal cells.

The percent of cells excluding dye in FA cultures declines slowly until 48 hours, then declines rapidly in an exponential fashion. The decline of percent cells excluding dye is much more rapid than the decline of total cells in culture. This suggests that some cells taking up dye must be living, and hence is evidence for a cell membrane defect.


100
50
20
10
5
2
1
Percent
PERCENT CELLS EXCLUDING DYE
(a)
TOTAL CELLS AS PERCENT
OF ORIGINAL INOCULUM
(b)
TOTAL VIABLE CELLS AS PERCENT
OF ORIGINAL INOCULUM
(c)
Control
Fanconi
With PHA
Without PHA
0 24 48 72 96 120 144 168
Hours in culture
DBL 705 5770

($T_{1/2}$) of about eight days. In cultures from the propositus, the same decline is seen until 48 hours. Thereafter the decline is more marked, with a $T_{1/2}$ (corrected graphically for the control rate) of about 1 3/4 days, or 42 hours. A similar procedure applied to cultures with PHA produces: control $T_{1/2}$ = 21 days (over the 7-day observation period); FA $T_{1/2}$ = 2 1/2 days, or 60 hours. The curves for total viable cells are composites and cannot be analyzed so simply.

Discussion

It seems fairly well established that the lymphocytes in circulating blood of most mammals and man may be divided into subpopulations [7, 102,111,145]. Their proportions and significance partly depend on the techniques used to detect them. Bender found early peaks in lymphocyte mitotic activity correspond to early-dividing radiosensitive cells, while later peaks correspond to a later-dividing less radiosensitive population [7]. From survival curves obtained from irradiated cells, Schrek and Stefani postulated the existence of two lymphocyte populations, one radiosensitive and the other radiorestant [111]. Variability of mitotic activity in the FA cultures could result from sensitivity of a cell subpopulation to some unknown insult.

On the other hand, Bender found he could not al-

ways detect his early/sensitive cell population in sequential cultures from the same cell donor [7]. Although absence of the early peaks in lymphocyte cultures from normal donors has never been seen in this laboratory, it may be an infrequently encountered normal phenomenon which has been accentuated in the FA cells.

It should be noted that while mitotic peaks were absent in some FA curves, mitotic activity was present. This could be a consequence of marked asynchrony in a cell population which would normally divide more or less synchronously. The possibility of division asynchrony is discussed further in connection with descriptions of experiments on the lymphocyte cell cycle.

Viability curves of control lymphocytes show generally higher survival of cells in vitro than was found by Schrek and Elrod [110], and Stewart and Ingram [126]. The net increase in cell number found by Stewart and Ingram in their study of PHA-stimulated lymphocyte cultures is not seen here; the difference is not explicable from the data.

Decreased viability in the FA cultures is more interesting. The known cytotoxicity of PHA [49] does not account for it, as the greatest decrease is found in FA cultures not exposed to PHA. This fact eliminates cell division as a precipitating event: normally

no dividing cells are found in lymphocyte cultures without PHA. Prior to 48 hours, cells in culture with or without PHA survive as well as control cells. It could be that a cytotoxic principle is released by the cells, the concentration of which rises to injurious levels by 48 hours. The principle could be a virus, as viral association with FA has been suggested [116, 128]. This possibility is examined by experiments described in a later section.

Another explanation for the decreased viability of FA cells is possible. The total number of viable cells, defined by dye exclusion, declines more rapidly with time than does the total number of cells in culture. Either some dead cells do not cytolyze, or dye uptake occurs in some living cells. These possibilities cannot be distinguished with the facts at hand, although many eosin-dyed cells were morphologically normal. The exclusion of eosin dye from a living cell is the result of an active process maintaining the selective permeability of the cell membrane. If living cells absorbed dye, they must have had a defect in their cell membranes or in the process maintaining its selective permeability.

The atypical mitotic index patterns and poor <u>in vitro</u> survival of cultured FA lymphocytes help explain the failure of many initial experiments. These phenomena were taken into account in subsequent experiments.

CELLULAR HEXOKINASE

A deficiency of cellular hexokinase in the cells of FA patients, as suggested by Lohr et al. [65,66] and Schroeder [113,114], could explain both the poor in vitro viability of Patty's lymphocytes and also the failure to obtain in vitro growth of her fibroblasts. The possibility of a hexokinase deficiency in Patty and her immediate relatives was examined by biochemical analysis of three different blood cell types. In addition, increased growth of fibroblasts was attempted using a specific augmentation in the growth medium.

Materials and Methods

Whole blood was obtained from the propositus and her immediate relatives in September 1968, and again in March 1970. Red blood cells (RBC) were obtained by sedimentation of the heparinized blood for 1/2 - 2 hours at room temperature. White blood cells (WBC) were obtained by centrifugation of the plasma overlying the sedimented RBC. Lymphocytes were obtained from separate blood samples by sedimentation and separation as previously described. Each separate sample was diluted with tissue culture medium without PHA or antibiotics, and sealed into a sterile 15 ml tissue culture tube retaining about 0.5 ml air volume. The coded tubes were immersed in ice contained in a foam-insulated shipping package, and then flown by commercial air-

craft to an independent laboratory for hexokinase activity assay on a "double-blind" basis. Seven hours elapsed between blood drawing and receipt of cell samples in the cooperating laboratory. Upon receipt the cells' viability was judged to be within normal limits.

RBC, WBC, and lymphocytes were assayed for hexokinase activity using the methods described by Valentine et al. [135] and Baughan et al. [6]. Briefly: A measured number of cells are lysed by freeze-thawing. In vitro, glucose-6-phosphate is quantitatively converted to 6-phosphogluconic acid upon the addition of crystalline glucose-6-phosphate dehydrogenase and diphosphopyridine nucleotide (NADP). In the reaction NADP is converted to NADPH, which is serially determined by absorption spectrophotometry at 340 mμ for one hour at 37°C. The results are expressed in "enzyme units", defined to be the number of micromoles of pyridine nucleotide converted per minute per 10^{10} cells at 37°C. Following the determinations, matching of samples with the appropriate results was made by interlaboratory comparison of the identification codes.

In September 1968 and again in July 1969 marrow fibroblast-like cells were obtained from the propositus. A sterile solution of glucose-6-phosphate was added to replicate fibroblast cultures at final concen-

trations of 1, 10, 100, and 1000 μg/ml. Previously marked cultures were observed at 24 hour intervals for 7 days to detect cell division and colony formation. Some replicate cultures were checked for viability by the dye exclusion test as previously described.

Results

A laboratory mistake prevented accurate decoding of the results for September 1968 blood cell samples. However, they were all reported to be within the normal range. The results of the March 1970 determinations are given in Table 5. The propositus has values within the normal range for all cell types. White cell values for the mother are elevated, those for the normal sib and the control are depressed.

There was no evidence of growth or division in any of the fibroblast cultures at any concentration of glucose-6-phosphate. In some cultures having higher concentrations, there was considerable accumulation of dense granules in many cells, and a subjectively-determined increase in cell detachment and loss.

Discussion

Deviations from normal hexokinase values seen in certain of the white cell samples are not considered biologically significant. They may be explained most simply as artifact produced by cell clumping, giving rise to counting errors when preparing the assays.

Table 5. Cellular Hexokinase

Subject	Relationship	Micromoles NADPH/minute/10^{10} cells: Red Cells	White Cells[1]	Lymphocytes
PH	Propositus	0.34	42.6	20.1
KH	Normal sib	0.20	18.2[2]	12.5
EH	Mother	0.25	77.8[2]	10.4
HH	Father	0.19	35.3	16.3
EC	Unrelated control	0.21	9.6[2]	16.8
	Normal range	0.17-0.31	24-58	Not established[3]

[1]All classes of WBC, including lymphocytes.

[2]These values, outside the normal range, may be artifact (see text).

[3]The range established for lymphocytic cells in chronic lymphocytic leukemia is 10-20 micromoles NADPH/minute/10^{10} cells.

Pure lymphocytes do not clump as readily as do granulocytes; they would be much less affected by this source of error. The RBC of the propositus have the highest RBC hexokinase activity of all the subjects. She is anemic, with a reticulocytosis; it may be assumed many of her circulating RBC are young. Syllm-Rapoport et al. report increased RBC hexokinase activity in young as opposed to older RBC [130], and this may explain the FA subject's high RBC hexokinase values. In any event there is no deficiency of hexokinase in any of the listed cell types from the propositus.

Hexokinase catalyzes the production of glucose-6-phosphate from glucose. Supplementing the fibroblasts' medium with that metabolite should have resulted in growth if the cells were deficient in hexokinase. With no evidence of growth in the augmented medium, it may be assumed that the fibroblasts also were not deficient in hexokinase solely. Poor growth of FA fibroblasts has been noted by others [46,77]; failure to obtain any growth at all is inexplicable. In this connection it might be noted that the FA subject's marrow cavities in vivo are either hypoplastic or aplastic, but do not show fibrotic changes. Perhaps her fibroblasts are deficient in growth potential in vivo as well.

Schroeder had proposed that FA might exist in two

forms [113,114]. One form was supposed to be hexokinase deficient and display increased chromatid exchanges; the other was supposed to be hexokinase normal with much less striking chromosome aberrations. The present finding, normal hexokinase levels accompanied by markedly increased chromatid exchanges, would be a third FA form on Schroeder's hypothesis. Other cases of FA with normal hexokinase activity are known, and that of Schuler et al. [117] has chromosome abnormalities differing somewhat from those reported by Schroeder and from those reported here. On the principle of Occam's Razor, a proliferation of varieties of FA seems unlikely. It is simpler to view hexokinase deficiency as an uncommon symptom in the FA syndrome, variable in both frequency and severity as are many of the other symptoms.

LYMPHOCYTE CELL CYCLE ANALYSIS IN VITRO

Elongated G_2 or S stages of the cell cycle have been suggested in earlier sections as events possibly associated with the large chromatid exchange frequency in cultured lymphocytes of the propositus. In an early experiment to obtain radiothymidine labelling patterns of her chromosomes, there was virtually no uptake of label by the chromosomes as much as 6 hours before mitosis. Both the questions and the observations indicated performing a complete cell cycle analysis on Patty's lymphocytes. This was done in October 1969 and also in March 1970. On the latter date similar studies were performed on all Patty's family members.

Materials and Methods

Pure lymphocyte suspensions were obtained from all family members by methods previously described. All cell batches were washed twice by centrifugation before they were planted in culture with PHA but without antibiotics. For these experiments the cells of each subject were cultured in several Erlenmeyer-type culture flasks. After 96 hours of culture, to obtain an approximation to "steady state" culture conditions, the contents of each subject's flasks were combined and thoroughly mixed, then redistributed to 9 ml culture tubes as identical cultures. At one hour in-

tervals thereafter tritiated thymidine[1] was added to successive cultures at a final concentration of 1 μCi/ml. The radioactive labelling compound either was removed after 10 minutes (pulse labelled cultures) or permitted to remain until culture harvest (continuously labelled cultures). Removal of the radiolabel from pulse labelled cultures was accomplished by adding non-radioactive thymidine to a final concentration of 10 μg/ml, then washing once by centrifugation in medium containing the same concentration of "cold" thymidine. This was followed by replenishment with pre-warmed fresh medium, and continued incubation. The "cold" thymidine added in the removal step was 5 times the usual concentration in medium, or enough to block further thymidine uptake [31, p. 561; 57]. Removal of the radiolabel from continuously labelled cultures occurred during the harvesting steps.

All cultures were harvested and cell fixation was accomplished 120 hours after planting, or 24 hours after the first cultures had been exposed to tritiated thymidine. After one-hour exposure to Velban, cultures were harvested and chromosome preparations were made by methods described previously. To minimize cell loss through osmotic disruption, the time in the hypotonic fluid was reduced by 15%. Concurrent sepa-

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[1]Thymidine methyl-T, sp. act. 5 Ci/mM. Amersham/Searle Des Plaines, Illinois.

rate determinations of cell viability were made as previously described.

Chromosome preparations were stained with LPO. This is inert to autoradiographic film and does not produce spurious silver grains [31, p.562;107]. After this, autoradiographic film was affixed to the slides in the dark using methods described by Pelc [89,90] and Doniac and Pelc [28]. Briefly: Under a very dim Kodak Wratten #1 safelight, AR.10 stripping film[1] was cut into 4x6 cm rectangles, removed from its backing under 70% ethanol, and floated emulsion-down on the surface of filtered de-ionized water at 18°C. The film was stripped under ethanol to eliminate grain artifact resulting from static discharges. After the film had absorbed water and flattened on the surface, it was raised from the water with a chromosome preparation slide. The cells on the slide and the emulsion of the film were in apposition, and the free ends of the film were allowed to overlap on the slide's reverse side [67]. Excess water was drained from the slides and they were placed in a light-tight chamber and gently dried with a current of filtered room-temperature air. After drying, the reverse sides of the slides were painted with a dilute synthetic resin to stabilize further the emulsion during later handling.

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[1]AR.10 Autoradiographic Stripping Film. Kodak Limited, London, England.

Dried slides were placed in light-tight boxes with capsules of silica gel[1]. The boxes were sealed with tape and stored in the dark at 4°C.

After 16 days exposure time, determined by prior experience and trial development of replicate sample slides, the autoradiographs were brought to room temperature slowly and developed. Under very dim safelight illumination, the slides were developed in Kodak D-19 Developer for 5 minutes at 18°C, followed by a rapid rinse in de-ionized water and fixation in Kodak Rapid Fixer with Hardener. After 10 minutes in the fixer, the slides were washed for 15 minutes under gently running tap water and dried under a gentle current of room air. Coverslips (#1 thinness) were affixed with Permount.

The stained and developed autoradiographs were examined microscopically at magnifications of 400 and 1250 diameters. The background frequency of silver grains was established by counting grains in cell-free portions of the slide. An eyepiece reticle defined an area equal to the average area of a well-spread metaphase cell (about 30 microns diameter).

Differential counts were made of labelled cells, unlabelled cells, labelled metaphase cells, and unlabelled metaphase cells, and the mitotic index of

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[1]"Driaire" Dessicator Humi-Cap. Driaire Inc., Norwalk, Connecticut.

each preparation was determined. To obtain the percentage of labelled cells, 300 - 500 cells were observed. To obtain the percentage of labelled metaphase cells an additional 100 - 200 metaphase cells were observed. The 95% confidence limits on the percentages were calculated by standard methods [25]. For each series of determinations a graph was made showing percentage of labelled cells as a function of radiothymidine addition time.

The curves of percent labelled metaphases from pulse labelled cultures (PLMp) were analyzed by the method of Quastler and Sherman [95] to obtain the lengths of the cell cycle periods. The G_2 period extended from the time of Velban addition to the 50% labelled point on the rising limb of the curve. The time between that point and the 50% labelled point on the descending limb of the curve was the S (DNA synthesis) period. The G_1 period could be estimated only; it extended from the 50% labelled point on the descending curve to the point at which the least labelling was next observed.

The curves of percent labelled cells from pulse labelled cultures (PLCp) were analyzed to obtain the fraction of labelled cells, and to determine the constancy of that fraction throughout the experiment. The appropriateness of linear regression was determined [76], and the regression of labelled cells on

time was obtained by standard methods along with the significance of any difference of the regression line from zero slope. The numerical values of mean labelled fractions were divided by viable fraction values determined mid-way in the study to obtain "true" fractions labelled of viable (cycling) cells.

Curves of percent labelled metaphases from continuously labelled cultures (PLMc) were analyzed as above to obtain a replicate estimate of the G_2 period length. PLMc curves should show a plateau of 100% labelling for all thymidine addition times earlier than about mid-S. Plateaus with less than 100% labelling provided an estimate of the fraction of cells reaching metaphase without uptake of radiolabel. Failure to reach a plateau was considered to be evidence of cell population kinetics not conforming to the requirements of the analytical method.

The curves of percentage labelled cells from continuously labelled cultures (PLCc) showed a rising portion and a plateau. Both were checked for linearity and regressions were obtained as before. The slopes of the rising portions of the curve provided the rate at which cells passed the G_1-S boundary. The time at which the plateau was reached was a measure of T_G-S = G_2+M+G_1. By substitution this yields G_1. Deviation of the plateau from 100% labelling was a measure of the fraction of non-labelling cells. Any significant dif-

ference between this fraction and the separately determined non-viable fraction of cells was a measure of the fraction of viable cells not using the radiolabelled thymidine.

Corrected estimates of the lengths of S and G_1 were obtained by the graphical method of Okada [86], using mitotic indices and G_2 times, with the estimates of T_G and the corrected "true" labelling fraction, as the four required parameters.

Counts of silver grains overlying metaphase chromosomes or labelled nuclei were not performed, but gross differences in label uptake among cells and between subjects were noted.

Results

PLM and PLC curves from pulse and continuously labelled cultures from the propositus and her family, and control subjects, are shown in Figures 12 through 15. A smoothed representation of Figure 12 is shown in Figure 16, and the regression lines obtained from Figure 15 are shown in Figure 17. Labelled fractions obtained from the data of Figure 13, and mitotic indices, are shown corrected for cell viability in Table 6.

The use of Okada's graphical analysis method applied to control cultures is shown in Figure 18. A summary of the best estimates of cell cycle parameters

Figure 12. Percent labelled metaphase cells in pulse-labelling experiments.

A. Control curves have rapidly ascending and descending portions, defining G_2 and S phases rather sharply. Both curves much more nearly reach 100% labelling than do the parents' curves in Figure 12 B.

B. The normal sibling has a labelling curve identical to those of control individuals. The parents' curves do not quite reach 100% labelling during the S phase, and are wider in extent than control curves during part of S or G_1 (see text).

C. Cultured lymphocytes of the FA case hardly show 50% of metaphase cells labelled at any time during the 24 hours prior to metaphase.

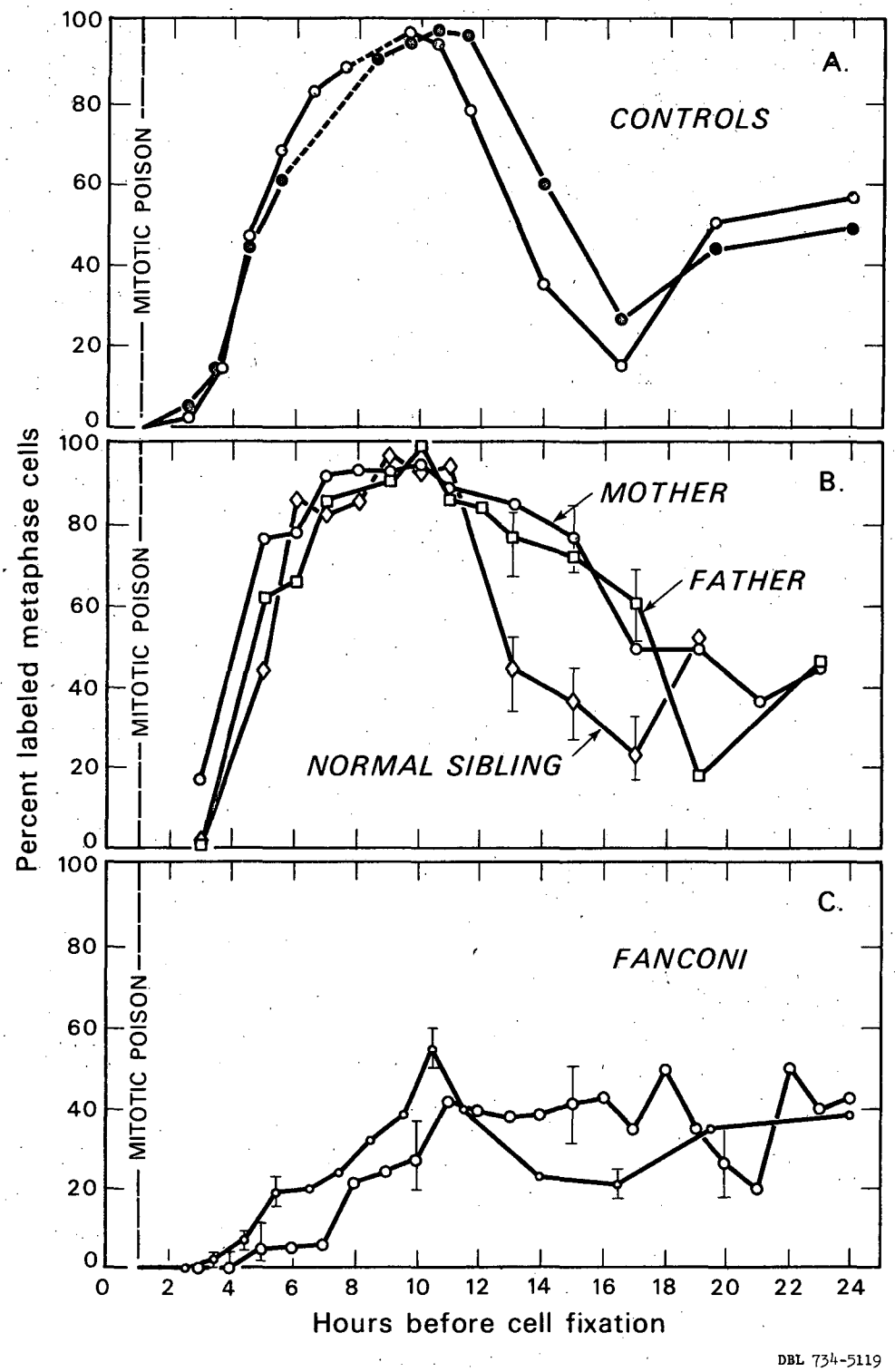
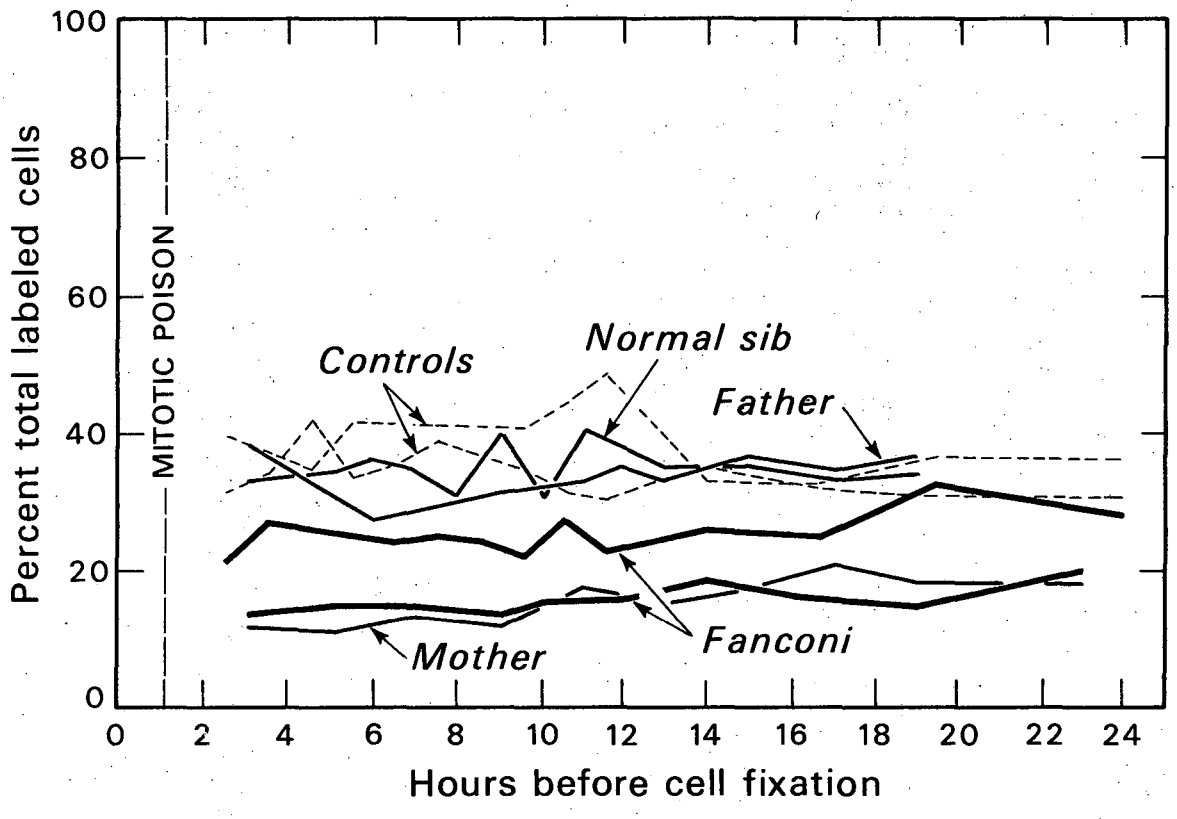

A.
CONTROLS
B.
MOTHER
FATHER
NORMAL SIBLING
C.
FANCONI
MITOTIC POISON
Percent labeled metaphase cells
Hours before cell fixation
0
20
40
60
80
100
2
4
6
8
10
12
14
16
18
20
22
24
DBL 734-5119

Figure 13. Percent labelled cells in pulse-labelling experiments.

The curves for all subjects approximate horizontal lines, indicating "steady-state" conditions in the cultures with regard to constancy of labelling fraction.


100
80
60
40
20
0
Percent total labeled cells
MITOTIC POISON
Controls
Normal sib
Father
Mother
Fanconi
0
2
4
6
8
10
12
14
16
18
20
22
24
Hours before cell fixation

DBL 734-5112

Figure 14. Percent labelled metaphase cells in continuous-labelling experiments.

A. Control curves reach 100% labelling later than do the comparable pulse-labelling curves, indicating a radiation-induced lag.

B. The parents' curves show a deficit in labelling at a time when control cells have achieved 100% labelling. A small fraction of the father's cells do not label even when exposed to radiothymidine for a period well in excess of a normal cell generation time. The normal sib's curve is the same as control curves.

C. A large fraction of metaphase cells in the FA culture does not become labelled, even when cells are exposed to radiothymidine for a period greatly in excess of a normal cell generation time.

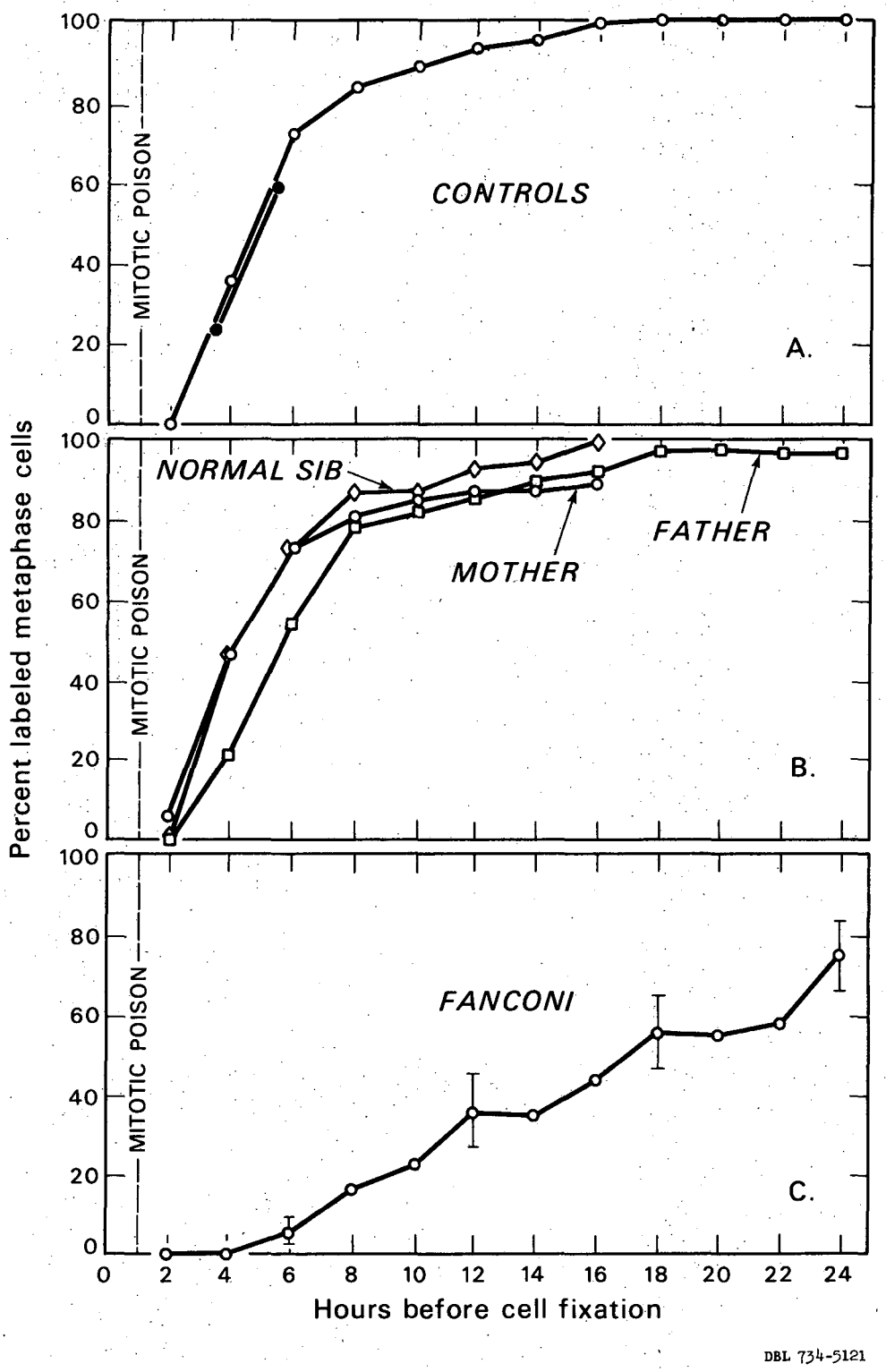
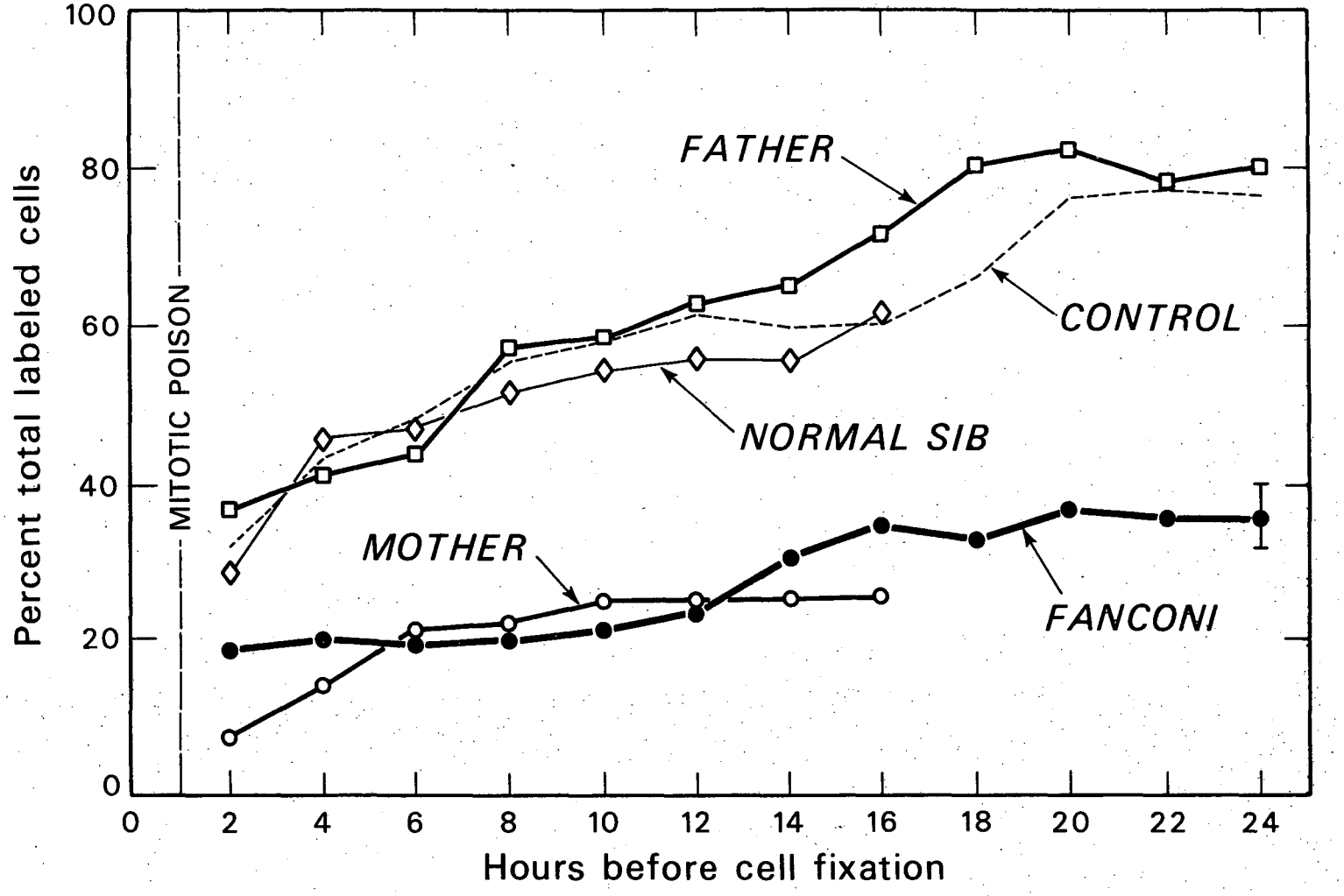

Percent labeled metaphase cells
MITOTIC POISON
CONTROLS
A.
NORMAL SIB
FATHER
MOTHER
B.
FANCONI
C.
0
20
40
60
80
100
0
2
4
6
8
10
12
14
16
18
20
22
24
Hours before cell fixation
DBL 734-5121

Figure 15. Percent labelled cells in continuous-labelling experiments.

The initial slope of these curves represents the rate at which cells enter S phase from G_1 phase; this rate is virtually the same in all subjects. The time at which the curve reaches a horizontal plateau is a measure of T_G-S; by subtraction of the known G_2 and M durations, the duration of G_1 phase may be estimated. After correction of the curves for dead cells, the FA and mother's curves have the same amplitude as the other subjects (see Figure 17).


Percent total labeled cells
100
80
60
40
20
0
MITOTIC POISON
FATHER
CONTROL
NORMAL SIB
MOTHER
FANCONI
0
2
4
6
8
10
12
14
16
18
20
22
24
Hours before cell fixation

DBL 734-5109

Figure 16. Smoothed representation of percent labelled mataphase cells in pulse-labelling experiments.

The data of Figure 12 are redrawn; curves for control and FA subjects are composites, and the curves are superimposed. The wide extension of the S phase portions of the mother's and father's curve is seen easily. The FA composite curve is grossly abnormal, and there is a suggestion of two labelling peaks.

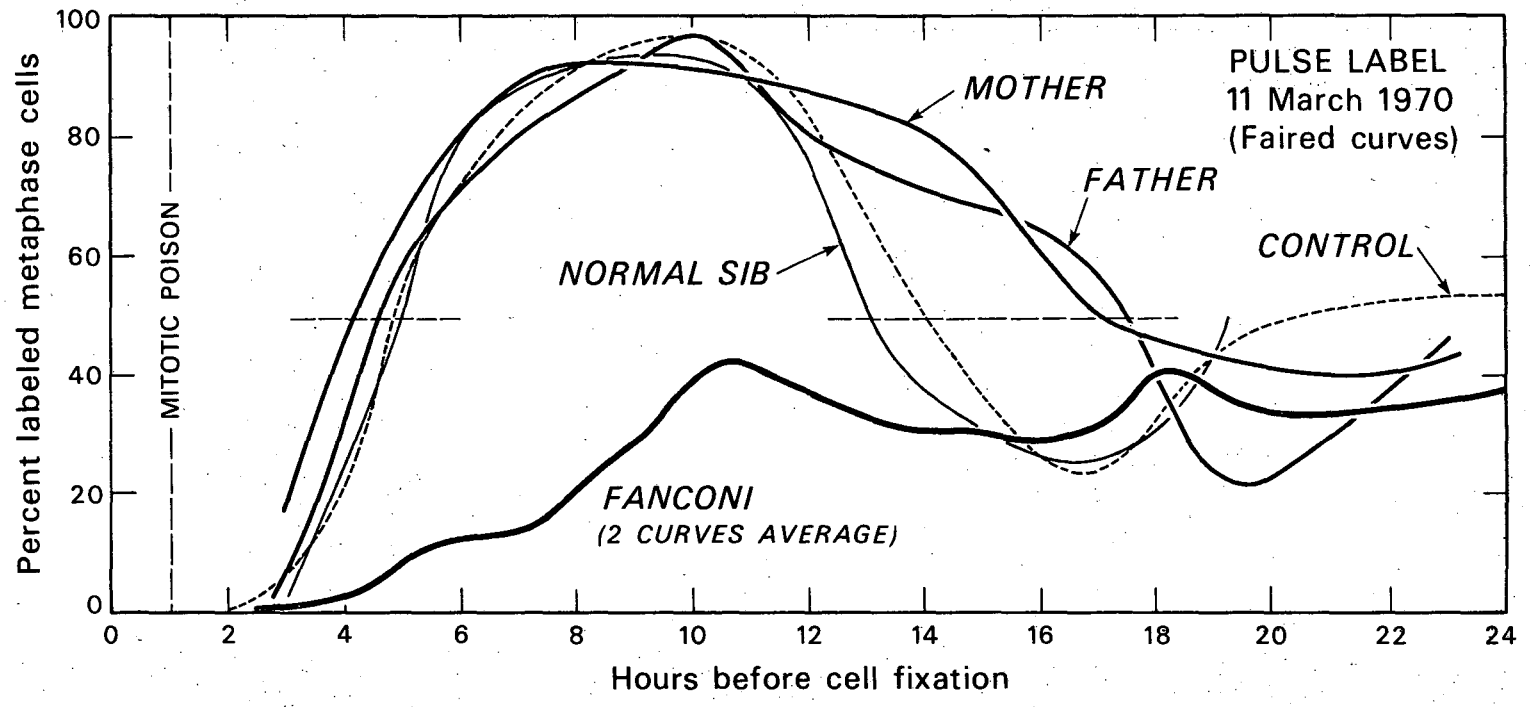

Percent labeled metaphase cells
100
80
60
40
20
0
MITOTIC POISON
MOTHER
FATHER
NORMAL SIB
CONTROL
PULSE LABEL
11 March 1970
(Faired curves)
FANCONI
(2 CURVES AVERAGE)
0
2
4
6
8
10
12
14
16
18
20
22
24
Hours before cell fixation
DBL 734-5117

Figure 17. Regressions on time of percent labelled cells in continuous-labelling experiments.

A. Regression lines from the data in Figure 13 are not significantly different from horizontal, showing "steady-state" conditions in the cultures with respect to the labelling fractions.

B. The regressions in A adjusted for the fraction of dead cells at the mid-point of the experiments. All have virtually the same amplitude.

C. Regression lines for both the rising and plateau portions of the curves in Figure 15.

D. The regressions in B adjusted for the fraction of dead cells at the mid-point of the experiments. All curves have virtually the same amplitude. The slopes of the rising portions in both mother's and FA curves are not significantly different from the control slope. It is interesting that their slopes are the least of all subjects, suggesting the possibility of reduced rates of entry to S phase from G_1 phase.

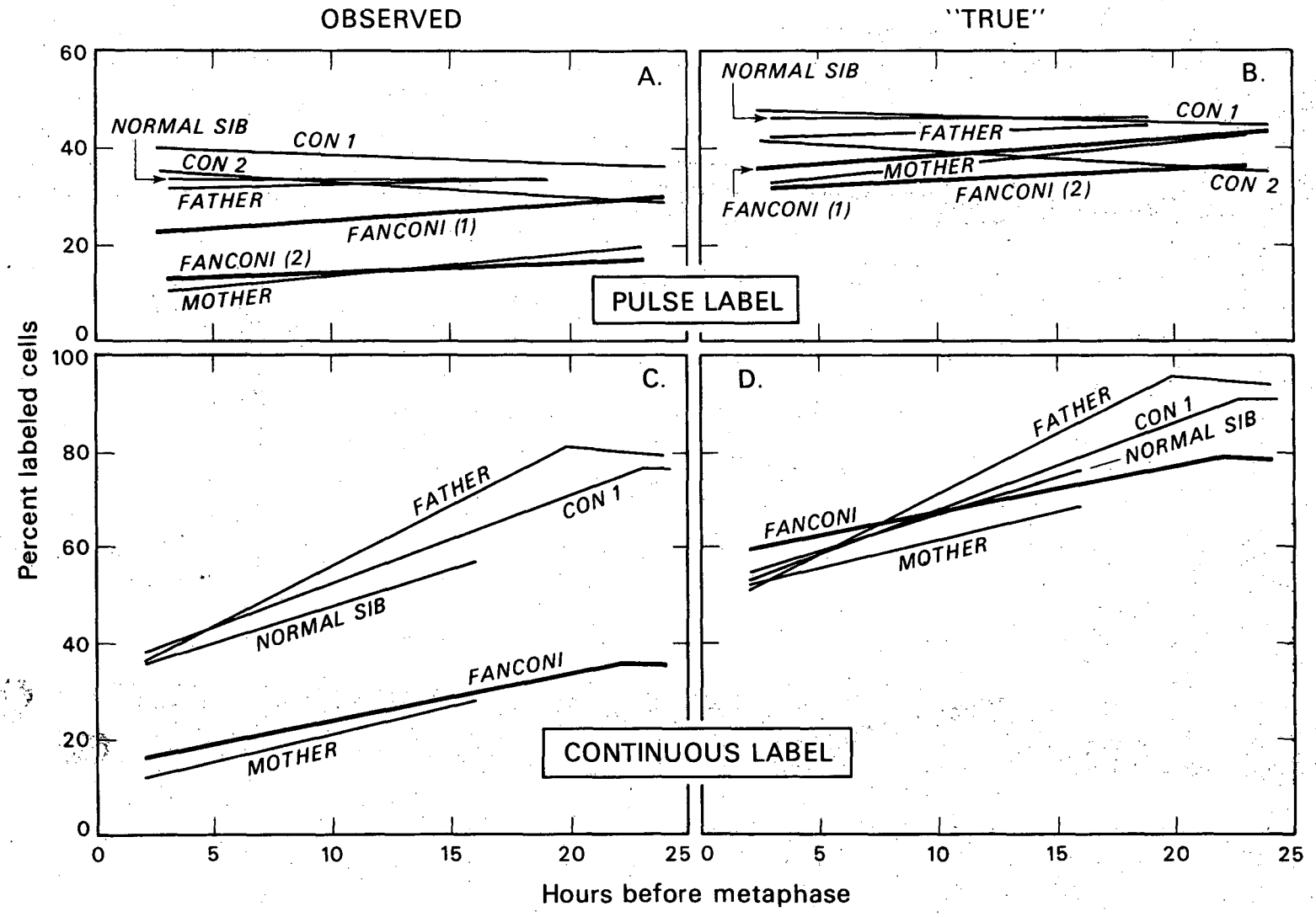

OBSERVED
"TRUE"
A.
B.
C.
D.
NORMAL SIB
CON 1
CON 2
FATHER
FANCONI (1)
FANCONI (2)
MOTHER
PULSE LABEL
CONTINUOUS LABEL
FANCONI
Percent labeled cells
Hours before metaphase
0
5
10
15
20
25
40
60
80
100
DBL 734-5122

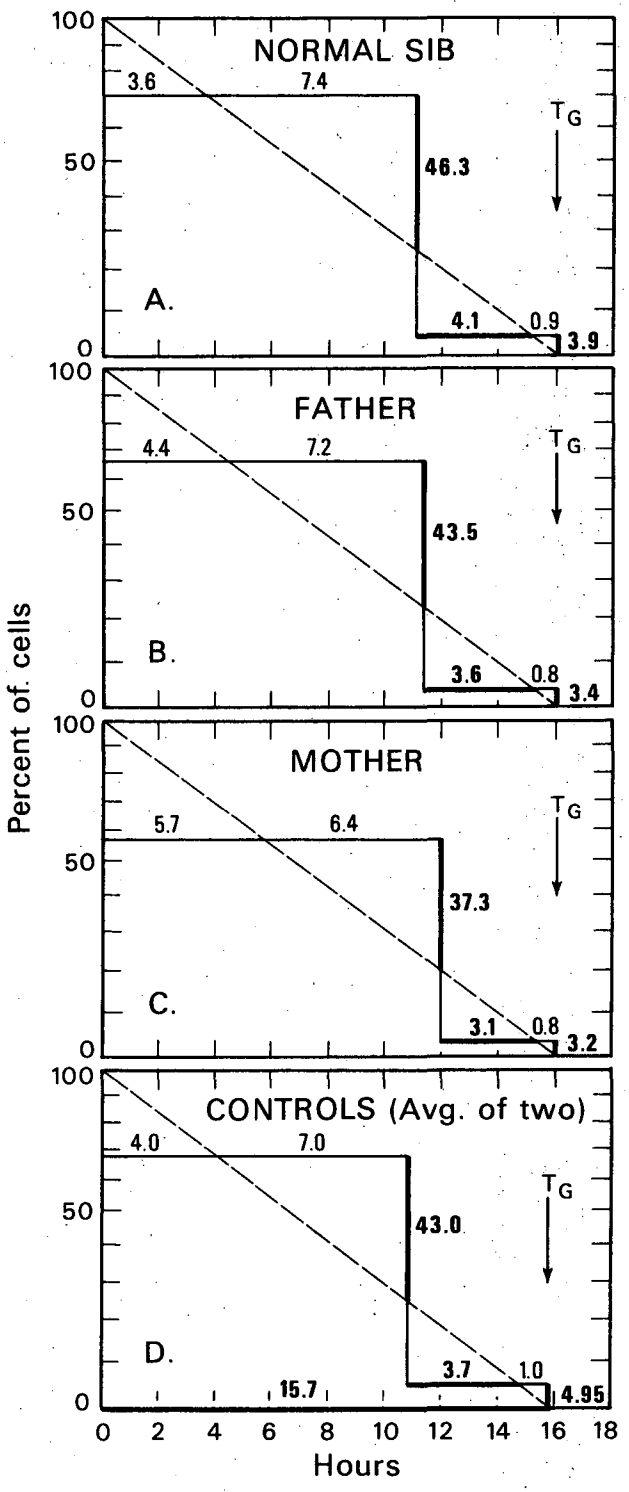
Table 6. Mitotic indices and labelled fractions of cultured lymphocytes, corrected for fraction of inviable cells.

	Mean Mitotic index (%)	Viable cells (%)	"True" mitotic index (%)	Observed labelled fraction (%)	"True" labelled fraction (%)
Control 1	4.1	83	4.9	38.9	46.9
Control 2	4.4	87	5.0	34.0	39.1
Average		85	4.95		43.0
Normal sib	2.9	75	3.9	34.7	46.3
Father	2.5	78	3.2	33.9	43.5
Mother	1.3	41	3.2	15.3	37.3
Average		64.7	3.43		42.4
FA case(1)	1.7	66	2.6	25.3	38.3
(2)	1.6	45	3.6	15.4	34.2
Average		55.5	3.15		36.3
MacKinney et al (Ref. 71)	<1%	--	--	--	40-45%

Mitotic indices and observed labelled fractions are means of all observations; percent viable cells are single mid-study values. "True" values are observed values divided by the fraction of viable cells.

Figure 18. Cell cycle component durations: Okada's method.

The ordinate is logarithmic, the abscissa linear. Reliably estimated quantities are shown by heavy numerals and heavy lines. Derived quantities are shown by light numerals. This graphical method of cell cycle analysis is used primarily to obtain good estimates of S and G_1 phases, for which Quastler's technique [95] provides spuriously high estimates.


NORMAL SIB
3.6
7.4
46.3
4.1
0.9
3.9
A.
FATHER
4.4
7.2
43.5
3.6
0.8
3.4
B.
MOTHER
5.7
6.4
37.3
3.1
0.8
3.2
C.
CONTROLS (Avg. of two)
4.0
7.0
43.0
3.7
1.0
15.7
4.95
D.
T_G
100
50
0
Percent of cells
0 2 4 6 8 10 12 14 16 18
Hours

DBL 734-5118

Table 7. Estimated durations of cell cycle components in cultured lymphocytes

	Raw Data Estimate[1]				Corrected Estimate				
	G_1	S	G_2	T_G	G_1[3]	S	G_2[1]	M[3,4]	T_G
Normal Sib	5.3	7.6	4.1	16	3.6	7.4[3] 7.35[5]	4.1	0.9[3] 0.62[6]	16[1]
Father	2.6 5.3[8]	12.8	3.6	19	4.4	7.2[3] 6.95[5]	3.6	0.8[3] 0.51[6]	16[2]
Mother	5.0	12.8	3.2	20	5.7	6.4[3] 5.97[5]	3.1	0.8[3] 0.51[6]	16[2]
FA	--- Not estimable ---				?	5.8[5]	?	0.5[6]	16 ?
Control (Av)	2.7 5.6[8]	9.3	3.7	15.7	4.0	6.75[5] 7.0[3]	3.7	1.0	15.7[1]
Cave (Ref.21)	4.6	9.5	3.5	17.6	---	---	---	---	---
Subjects (Range)[7]	---	---	---	---	3.6-4.4	6.4-7.4	3.1-4.1	0.8-1.0	15.7-16.0

(Footnotes on following page)

Table 7. Continued

[1]From PLMp curves in Fig. 12, and PLMc curves of Fig. 14

[2]Assumed: Value of normal sib from Fig. 12

[3]By Okada's method, using T_G shown

[4]From corrected mitotic index

[5]From corrected average labelling index x indicated T_G

[6]From corrected mitotic index x indicated T_G

[7]Without FA values, and without mother's G_1

[8]From PLCc curves in Fig. 15, and calculation

is given in Table 7.

From the curve of PLMp in Figures 12 and 16 it is seen that neither the mother nor the father reach the maximum labelling of the control curves. The widths of the curves at 50% labelling are extended, indicating greater variability among S periods than found in controls. The normal sib presents a curve virtually identical to that of the controls. The FA propositus has remarkable and highly unusual curves which hardly reach the 50% labelling level, and then only briefly.

The PLCp curves in Figure 13 are essentially horizontal lines, without significant deviation of individual data points. This is indicative of "steady state" conditions in all cultures, in the sense of labelling index time constancy [38]. PLCp curves for the normal sib and the father are virtually the same as controls: all show labelling indices between 34 and 39 percent. The curve for the mother is virtually the same as those of the FA propositus, both yielding reduced labelling indices of 15 to 25 percent. Correcting all data for the fraction of inviable cells produces similar labelling indices in all subjects but the FA propositus. Her labelling index remains about 15% lower than the mean value for the other subjects.

As expected, the G_2 intervals obtained from the PLMc curves in Figure 14 are the same as those found from the PLMp curve. Again however, the curve of PLMc

for the propositus is remarkable and unusual. The labelling index never approaches 100%, though the curve spans a period in excess of the controls' generation time. The remainder of the curves approximate the 100% labelling level 6 hours later than expected on the basis of the PLMp curves. This is a measure of the lag produced in either or both of S and G_2 through continual exposure to radiation. PLMc curves for the mother and father do not reach 100% labelling. At all times after reaching a plateau, these curves are approximately 5% below the control index (100%). This must represent a proportion of cells which reach metaphase without utilizing the exogenous radiolabelled thymidine.

In Figure 15, PLCc curves for the mother and FA propositus are the same, and show lower labelling indices than the other subjects. As before, correcting the indices for the frequency of inviable cells yields curves which are virtually the same in all subjects. The plateaus of the curves for the control and the father, close to 80% labelling, are an independent estimate of the viable (or cycling) cell fraction; these values agree fairly well with those obtained by dye-exclusion (Figure 11; Table 6). The plateaus appear at 20 hours. Subtracting the 6 hour radiation-induced lag gives 14 hours as an estimate of T_G-S. The estimated durations of G_2 and M (Table 7) are subtracted,

yielding 5.3 and 5.6 hours as estimates for the G_1 duration in the father and control respectively. The normal sib's G_1 cannot be estimated directly, but is likely to be similar. Both the FA case and her mother show anomalous plateaus in, and reduced slopes of, their PLCc curves. Assuming linear labelling increases (Figure 17) and labelling of all viable cells (Table 6), then either or both of G_1 and G_2 in these subjects must be lengthened. Their anomalous plateaus could indicate radiation-induced blocks in those cycle phases.

Discussion

The cell cycle of human lymphocytes *in vitro* and *in vivo* has been investigated by others [4,10,21,61, 122,131], and the uncorrected normal cell cycle times given here agree well with the raw data provided in those reports. The corrected normal labelling indices from PLCp curves agree well with those found by MacKinney *et al*., but their mitotic indices were lower than those found in this study [71]. The one-hour Velban mitotic index was used here, and is considered little different from the instantaneous mitotic index. Prior experience indicated less than 20% increase in MI over one hour exposure to Velban; the action of the drug is not quite immediate [69].

The PLMp, PLMc, PLCp, and PLCc curves for the nor-

mal sib are virtually identical to those of the controls. Her cell cycle times obtained by Okada's method therefore correspond well to those of the controls.

Cell cycle times for the mother and father are not so easily estimated from the PLMp curves. The requirement of small variability in S and G_1 times is not met, as shown by the lower-than-control peak labelling in S and the wide extension of the curves [38]. However the father's T_G may be estimated from the PLCc curve after correction for the radiation-induced lag shown by the PLMc curves, and proves to be close to that of the normal sib. Lacking this sort of data for the mother, a similar calculation cannot be made. However her PLMp curve is nearly the same as the father's, and there seems no reason to believe her T_G is grossly different. Using the 16-hour T_G of the normal sib to estimate the T_G's of mother and father, their corrected cell cycle times are found to match the control with one exception: the mother's G_1 is almost 30% longer than that of the other subjects. The significant observations then are increased variability in either or both of S and G_1 in both parents, and a lengthened G_1 in the mother's cells.

Cell cycle component durations determined by Okada's method are very sensitive to the value of T_G used. Since T_G is obtained from PLMp curves, and is

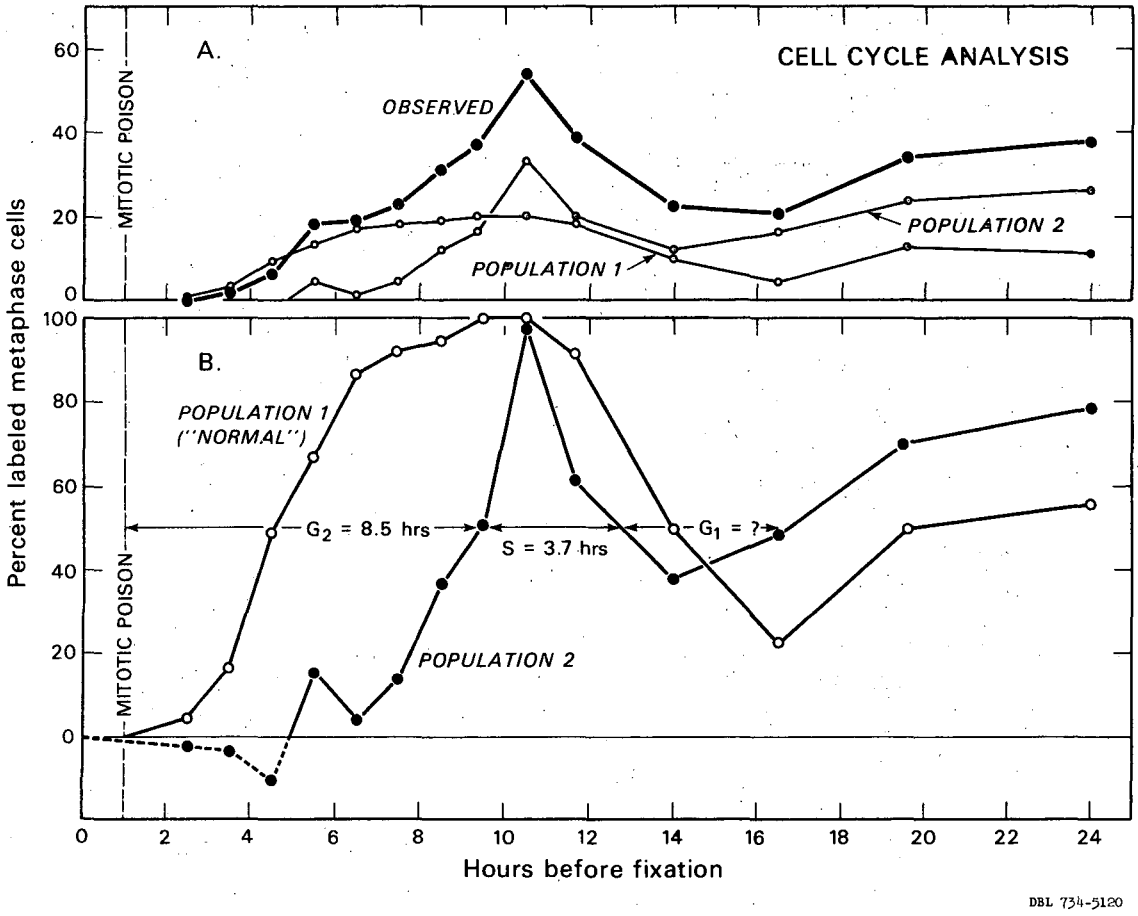
an approximation, all cell cycle times given in Table 7 must be considered estimates of the absolute values. However comparisons among the subjects are reasonably made since all T_G's are assumed equal or nearly so. The mother's long G_1 could be an artifact of the method, but this likelihood is reduced by the observation that her other cell cycle times are not greatly different from those of the other subjects. Reasonable upward adjustments of the other components still leaves the mother's G_1 the longest among the subjects.

The PLM curves of the propositus virtually defy analysis. A curve similar to her PLMp curve was observed by Baserga and Weibel in ascites tumor cells from fasted, but not from well-fed, mice [5]. There is no reason to believe the cells were starved in culture: the medium used was rich in nutrients, and other subjects' cells produced normal or nearly normal curves. In any event, the curve of PLMc never reaches 100% label in well over a normal cycle time. It is inescapable that the FA culture contained a large proportion of cells (between 40% and 60%) which did not incorporate the exogenous radiolabelled thymidine into their chromosomes. In addition there must be very great variability in the cell cycle times of the labelling population. After correcting the PLCc and PLCp curves of the FA case for dead cells, they are fairly close to those of the other subjects. A be-

lievable but perhaps naive extrapolation from this data suggests the FA subject's average T_G may be close to normal. If so, it is possible to hold that the labelling cells shown in the PLMp curves consist of two or more populations having the same average T_G, but a markedly lengthened G_2 in one. Bianchi and deBianchi claim this is a normal feature of a very small fraction of human lymphocytes [10]. Both PLMp curves contain a suggestion of a plateau shortly prior to mitosis; this may be interpreted as evidence for a population of cells with a normal G_2. Accepting this putative plateau as 100% labelling for the presumed normal fraction of cells, and graphically subtracting a normal curve of this amplitude from the first of the PLMp FA curves, reveals the component curves shown in Figure 19. The presumptive abnormally labelling population then is seen to have a lengthened G_2 and a shortened S. The same operation on the second FA PLMp curve gives a similar but less striking result.

After correcting for inviable cells, the "true" labelling fraction of propositus's cells is about the same as those of the other subjects. This implies a relatively normal S/T_G relationship. Yet from the PLM curves, about 50% of metaphase cells do not label, implying that the S/T_G relationship cannot be normal. The data are incompatible, and can be reconciled only if one or the other was erroneously derived. The PLM

Figure 19. Putative components of percent labelled metaphase curves of the FA propositus. The early rising portion and the early plateau on the observed data curve are assumed to represent a normally labelling cell population, with a labelling fraction at 7 1/2 hours the same as in controls. The control curve is drawn in proportion to that value, and is shown as Population 1. In turn, these data points were subtracted graphically from the observed data, yielding the curve shown as Population 2. The upper graph shows both Population 1 and 2 in real proportion to the observed data. The lower graph compares Population 1 and 2 redrawn in proportion to 100% labelling for their maximum labelling data points. The putative Population 2 then is found to have lengthened G_2 and shortened S phases.


A.
CELL CYCLE ANALYSIS
OBSERVED
POPULATION 2
POPULATION 1
MITOTIC POISON
B.
POPULATION 1
("NORMAL")
G_2 = 8.5 hrs
S = 3.7 hrs
G_1 = ?
POPULATION 2
MITOTIC POISON
Percent labeled metaphase cells
Hours before fixation
DBL 734-5120

curve must be correct, as the metaphase cells must have been living; the "true" labelled fraction must be incorrect. It is derived from the observed labelled fraction, which cannot be disputed, and the percentage of "inviable" cells defined by dye-exclusion. This ordinarily is a reasonable criterion, but must be inappropriate in this case: some living cells must have taken up dye and been misidentified as dead. If a higher fraction had been identified as living, the "true" labelling fraction would have been lower and hence more in accord with the PLM data. This seems good indirect evidence for failure of the selective permeability property of the cell membranes of a fraction of the propositus' lymphocytes. This effect cannot be ruled out in the mother's case, in light of her reduced labelling indices. However it seems unlikely to be a significant feature of the mother's cells, as her PLM curves are close to normal.

The slopes of the PLCc curves are the same in all subjects within the precision of the data (Figure 17). Thus the rate of passage of cells from G_1 to S must be virtually the same in all subjects' cells, including those of the FA case.

The proposed existence of subpopulations within the fraction of cells accepting label is speculative. But the existence of a subpopulation of cells which fails to label at all is not speculative, and requires

some explanation. Under certain non-normal conditions thymidine labelling is not an accurate indicator of DNA synthesis [23,134]. It is held widely that cells cannot reach mitosis without prior DNA synthesis [26, 63], and thymidine is required in DNA synthesis. The chromosomes of the unlabelled FA metaphase cells appeared perfectly normal; hence the FA cells must have manufactured thymidine from unlabelled precursors, probably by methylation of uridine [23]. This may be a normal and exclusive process in some cells, as Alpen and Johnston found a small fraction of normal canine marrow cells in vivo failed to label with radiothymidine [3]. But the data presented here show this does not occur in cultured normal human lymphocytes. It seems then that the unlabelled cells must have utilized atypically only one of two normally available DNA synthesis pathways.

The putative unused pathway involves the enzyme thymidine kinase, which catalyzes creation of thymidine monophosphate from exogenous thymidine [23]. A deficiency of thymidine kinase in the unlabelled cells is suggested, a possibility which has been investigated through experiments reported in a later section.

Alternatively the cells might not be able to transport thymidine across their cell membranes. Breslow and Goldsby invoked this explanation for their failure to obtain radiothymidine labelling in mutant

Chinese hamster cells having normal thymidine kinase activity, but they did not try to confirm this by experiment [13].

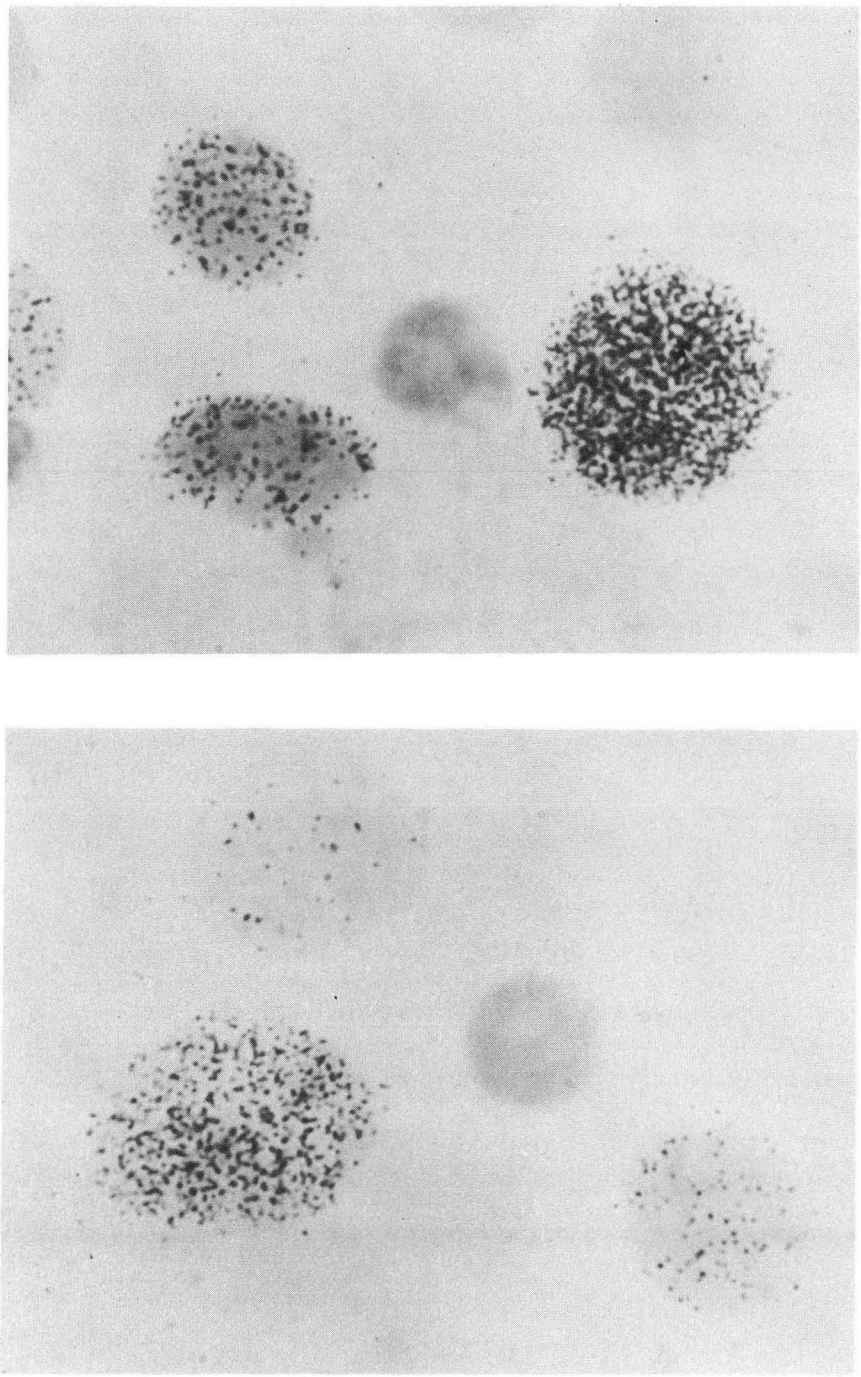
The mother's PLM curves show labelled cells at a time in late G_2 when all other subjects' cells showed no label. The amount is small, only 5%; but this may be evidence either for "repair" synthesis of DNA or a short G_2 in some of the mother's cells.

The majority of cells from both the mother and the FA propositus were labelled about as densely as those of all other subjects; but a small fraction of their cells showed very dense label which completely obscured the nucleus. This fraction amounted to about 10% in the mother, but only about 1% in the FA case. Subjectively the densely labelled cells appeared to have about 10-fold more label than the normally labelled cells; they are shown in Figure 20. Arguing both from the density of label and from the time at which they are first seen (4 - 10 hours prior to metaphase), these cells cannot have transitted the S period twice. "Repair" DNA replication in these cells is a possibility, of course, but this and any other explanations are both speculative and very limited by the facts at hand.

It may be argued that radiation to the cells received from the radiothymidine in the medium was responsible for the results described. Radiothymidine

Figure 20. Differentially labelled cells found among cultured lymphocytes of the FA case and her mother.

Continuous labelling experiments were conducted in such a way as to produce relatively light maximum labelling in the cultured cells. Both the FA case and her mother had a minority of cells which labelled heavily, even when the label was available only 4-10 hours before fixation. The photographs show heavily labelled cells, normally labelled cells, and unlabelled cells from cultures exposed to radiothymidine about 6 hours before fixation. The upper photograph is of FA cells, the lower of mother's cells. No similar heavily-labelled cells were found in cultures of controls or the normal sib. Magnification = 1820x.



XBB 734-2452

in the medium at a continuous concentration of 1 μCi/ml delivers about 5r/day to nuclei which take up the label [134, p.79]; a smaller dose would be delivered to pulse labelled cells. This is 25% of the dose required to inhibit growth [134], and control cultures showed none of the effects seen in the FA cultures. The FA cultures had normal rates of entry to S from G_1. Generally, it does not seem likely that radiation was responsible for the results. It remains possible that some cells of the mother, father, and propositus have a thymidine metabolism process which is exquisitely sensitive to radiation.

It may be argued further that the medication received by the FA propositus was responsible for the findings reported here. Both the mother and the father are shown to have a small population of non-labelling cells; aside from the mother's Diabinase neither is receiving medication. Further, preliminary experiments using lymphocytes from patients with a variety of hematological conditions, receiving either or both of prednisone and hematinics, show no such changes; no unlabelled cells were found in a single normal culture to which Delatestryl had been added [69]. Schmid et al ruled out steroids as a cause of chromosome changes in cultured FA cells [109]. It seems unlikely that the patient's medications produced the results described.

Detection of the heterozygous "carrier" of the

abnormal gene is desirable in dealing with genetic diseases. In FA, the parents are presumed to be the carriers of a recessive gene. Dosik et al. and Todaro et al. have used susceptibility to viral transformation of cultured cells in a test to detect presumptive FA heterozygotes [29,133]. Transformation of cultured cells by a tumor virus is greater among presumptive heterozygotes, and greatest among FA cases. The test must be performed in a laboratory equipped for viral studies, and is not specific for FA [92].

The differences in radiothymidine labelling indices among normals, parents, and an FA case shown here could be used similarly as a test for latent FA or to detect the heterozygous condition. A single 3-day lymphocyte culture would be exposed continuously to an appropriate level of radiothymidine. Eighteen to 24 hours after label addition a single autoradiograph of harvested cells would be made and examined for unlabelled metaphase cells. Normal cells would have no (or very rare) unlabelled metaphases, heterozygotes would have several percent, and FA cases would have a large percentage. This proposed test might not be specific either, as arrested DNA synthesis has been observed in a fraction of marrow cells from a case of erythroleukemia [24]. However the test would be simple enough to be within the capability of most clinical laboratories, even the smallest.

THYMIDINE KINASE DETERMINATIONS

Failure of a large fraction of FA lymphocyte metaphase cells to label with tritiated thymidine suggested the possibility of a defect in the cells' thymidine metabolism. A much smaller fraction of cells from both parents of the propositus also failed to label, suggesting the defect might be a direct manifestation of the recessive gene believed to be responsible for FA. Preformed thymidine incorporation into cellular DNA is catalyzed by the enzyme thymidine kinase [23]; the failure of some cultured cells to label with exogenous thymidine could be evidence for a population of cells deficient in this enzyme. Accordingly purified lymphocyte preparations were obtained from the propositus and members of her immediate family in February 1971, and these were assayed for thymidine kinase activity by an independent laboratory.

Materials and Methods

Using the methods described earlier, lymphocyte cultures were established from the FA propositus, her immediate family, and from an unrelated normal individual. These were cultured for 60 hours with PHA but without antibiotics. Total cell number and cellular viability were determined as described, the cells were washed twice by centrifugation in physiological saline solution, and the pellet of cells was quick frozen over

dry ice and stored in liquid nitrogen. The samples were submitted the same day to the cooperating laboratory, which assayed them for thymidine kinase activity using the method of Rabinowitz et al. [97] modified for very small samples.

Briefly: Thawed cells were disrupted sonically in pH 7.5 Tris buffer; supernatant fluid was obtained by centrifugation for 20 minutes at 10,000 g at 4°C. After addition of reactants, the supernatant fluid contained: 0.1 M Tris buffer, $MgCl_2$, K_2HPO_4, phosphoenolpyruvate, pyruvate kinase, ATP, mercaptoethanol, and high specific activity TdR-H^3. Following incubation of the mixture at 37°C for one hour, the entirety of each sample was chromatographed in isobutyric acid/EDTA/NH_4OH solvent for 16 hours, followed by scintillation counting of the eluted radioactive spots in 5 ml volumes. The sums of the recovered radioactivity in the mono-, di-, and triphosphates of thymidine was a measure of thymidine kinase activity in the entire cell sample. Results were reported as counts per minute (cpm) per 10^6 viable cells.

Results

The results of the thymidine kinase activity assays are shown in Table 8. All samples are in the range of 1000-2000 cpm per 10^6 cells except that of the propositus which has 68% more activity than the highest

Table 8. Thymidine kinase activity in cultured lymphocytes

	Number of cells per sample ($x10^6$)	Transformed cells (%)	Viable Transformed cells (%)	Number of viable transformed cells in sample ($x10^6$)	Total radio-activity (cpm)	cpm/10^6 viable transformed cells
Propositus	5.34	100	61	3.26	54,262	16,645
Normal sib	11.56	92	100	10.64	111,878	10,515
Father	10.49	100	100	10.49	46,675	4,449
Mother	8.87	89	100	7.89	41,347	5,240
Normal Control	13.8	100	100	13.8	91,105	6,597

of the other samples.

Discussion

That the FA patient should have much greater thymidine kinase activity than normal is surprising. The results are very sensitive to the accuracy of the original cell number and cell viability determinations, but this cannot account for the large and similar kinase activities found in the propositus and her normal sib. Both parents and the normal control were individuals in the 40-50 year age range, while the normal sib and the propositus are in their teens. There is then a possibility that cells from younger individuals show greater thymidine kinase activities in culture than those from older persons, as could be inferred from Bach's comments [4].

In any event there is no evidence for thymidine kinase deficiency in any of the cells studied. The failure to obtain labelling in all the cells of the propositus and her parents during cell cycle studies cannot be explained by a deficiency of this enzyme. Further, the markedly increased uptake of label seen in those studies in some of the mother's cells cannot be explained by excessive thymidine kinase activity.

As mentioned earlier, failure to obtain radiothymidine labelled cells in the presence of adequate thymidine kinase activity could be due to a membrane

transport defect [13]. It may be recalled that a membrane permeability defect, or defect of the metabolic process maintaining the membrane's selective permeability, was suggested in the earlier cell viability studies. The normal thymidine kinase activities found in all subjects' cells leave a thymidine transport defect, and hence possibly a membrane defect as a putative feature of FA.

CROSS-INCUBATION OF FA AND CONTROL CELLS

The earlier observation of reduced *in vitro* viability of FA lymphocytes, coupled with others' suggestion of viral involvement in FA [116,128], prompted repetition of the cross-incubation experiments previously reported [11,55,91].

Materials and Methods

In February 1970 white blood cells were obtained from the propositus and a normal individual, and pure lymphocyte suspensions were prepared as previously described. Cell-free plasma was obtained from both individuals by centrifugation of whole blood followed by membrane filtration. Culture medium containing both PHA-M and 10% v/v of either plasma was prepared. The lymphocytes were washed twice by centrifugation in physiological saline solution, and planted into the culture media at concentrations of 10^6 cells/ml. Replicates of four types of culture were prepared: Control cells in control plasma, FA cells in FA plasma, control cells in FA plasma, and FA cells in control plasma.

After 60 hours of incubation as previously described, some control cells in control plasma and FA cells in FA plasma were centrifuged for 5 minutes at 1000 g. The supernatant medium was removed aseptically and saved. The cells were washed twice by centrifugation in physiological saline. The salvaged FA medium

was placed onto control cells, control medium was placed onto FA cells, and incubation of all cultures was continued for 16 hours. At the end of this 76-hour period, all cultures were exposed to Velban for 1 hour as described, and harvested. Air-dried chromosome preparations of FA cells were unsatisfactory, and microscopical determination of chromosome aberration frequencies was performed on squash preparations in LPO stain.

Results

The results of the aberration frequency determinations on ordinary and on cross-incubated cultures are shown in Table 9. Under any culture conditions FA cells have a higher aberration rate than do control cells in ordinary cultures, as expected. "Refreshing" the FA cells by incubation in medium previously occupied by control cells did not reduce the aberration frequency significantly.

Control cells incubated in medium containing fresh FA plasma do not have an elevated aberration frequency. However, control cells incubated in medium occupied for 60 hours by transformed FA lymphocytes show a 7-fold increase in the frequency of simple chromatid breaks. Compared to the frequency found in ordinary control cultures, the elevation is significant ($t=2.14$, P less than 0.05) [25,123,125]. In the same culture

Table 9. Chromosome aberration frequencies in cross incubated lymphocytes of FA and normal individuals.

TYPE OF CROSS-INCUBATION [1]	CHROMATID ABERRATIONS										CHROMOSOME ABERRATIONS						
	BREAKS		CHROMATID EXCHANGE C/C						ISO/CD								
			INTERCHANGE		INTRACHANGE				INTER	INTRA					DELETIONS		
					INTER-ARM		INTRA-ARM										
	CTD	ISO	SYM	ASYM	SYM	ASYM	SYM	ASYM			DIC	RING	TRI	ACR	I.D.	TERM	GAPS
Control cells; Control plasma [2]	0.5 [4]																1
FA cells; FA plasma [2]	4	1	5	1					1		2						6
Control cells; FA plasma [2]	1																1.5
FA cells; Control plasma [2]	9	1	4	2					1								4
60-hr control cells; 60-hr FA medium [3]	3.5 [4]																3
60-hr FA cells; 60-hr control medium [3]	6		3	5					1		1		1				9

[1] *200 cells examined in control cell cultures; 100 cells examined in FA cell cultures*

[2] *76 hours total incubation*

[3] *60 hours incubation before switching cells, followed by 16 hours further incubation before fixation*

[4] *The seven-fold increase in frequency of chromatid breaks is significant (t = 2.24; P = 0.013, one-tailed)*

DBL 734-5125

the frequency of gaps is elevated as well, but this is not significant ($t=1.43$, P greater than 0.05).

Discussion

The cross-incubation experiments of others have used fresh FA plasma-containing medium [11,55,91]. The negative results reported by these workers is in accord with results obtained here in exactly comparable experiments. The elevated frequency of simple chromatid breaks found in control cells cross-incubated late in culture supports the contention made in a previous section that a cytotoxic principle was released by FA cells after 48 hours in culture. The principal may not be strictly cytotoxic to normal cells. Subjectively the cultures appeared healthy without evidence of excessive cell death or growth difficulties. The present experiments give no clue to the nature of the principal released, but they do suggest examination by electron microscopy of FA cells after a few days in culture.

ELECTRON MICROSCOPY OF LYMPHOCYTES

In a preceding section it was supposed that a toxic principle appeared in the FA lymphocyte cultures, and this might be an explanation for the increased cell death found there. In the cross-incubation experiments described earlier some agent was present in culture medium conditioned by FA lymphocytes which increased the frequency of simple chromatid breaks in control cells. Nichols has shown that viruses can cause chromosome breakage [78], and viral involvement in FA has been suggested [128]. Accordingly, electron microscope examinations of lymphocytes from the FA propositus were performed in February 1970.

Materials and Methods

Pure lymphocytes, and lymphocytes from 72-hour PHA stimulated cultures, were obtained by methods already described from both the FA propositus and a normal individual. The cells were washed once by centrifugation in fresh medium, then fixed for 12 hours in 2.5% w/v glutaraldehyde in 0.1 M sodium cacodylate. Following three washes in 0.1 M sodium cacodylate, the cells were post-fixed for 1 hour in 1% w/v osmium tetroxide in 0.1 M sodium cacodylate. The samples were dehydrated in a series of increasingly concentrated alcohols terminating in 100% ethanol, then pelleted by centrifugation

and embedded in Epon 812[1] (resin: catalyst = 1:1). The cells were sectioned at 600-800 nanometers in the Sorvall MT-2 ultramicrotome[2]. Sections were stained for 30 minutes in a saturated aqueous solution of uranyl acetate, and for 10 minutes in Reynold's lead citrate stain [101]. After mounting on collodion filmed grids in the usual manner, the cells were examined in a Hitachi HU-11 microscope[3].

Cells selected under low power (5000 diameter magnification) were observed and photographed at successively higher magnifications up to 80,000 diameters. Each cell was examined for deviations from normal ultrastructure and for the presence of viral or proviral bodies.

Results

Electron micrographs of normal and FA cells are shown in Figures 21 and 22. The morphology and ultrastructure of normal cells, both fresh and from 72-hour cultures, conforms to descriptions given in the literature [20,46,58]. The same is true for the fresh FA lymphocytes.

The lymphocytes from 72-hour FA cultures show both

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[1]Epon 812 Resin. Ernest Fullam, Inc., Schenectady, New York.

[2]Ivan Sorvall, Inc. Norwalk, Connecticut.

[3]Hitachi, Ltd., Tokyo, Japan.

Figure 21. Electron micrographs: comparison of fresh lymphocytes from FA and normal individuals.

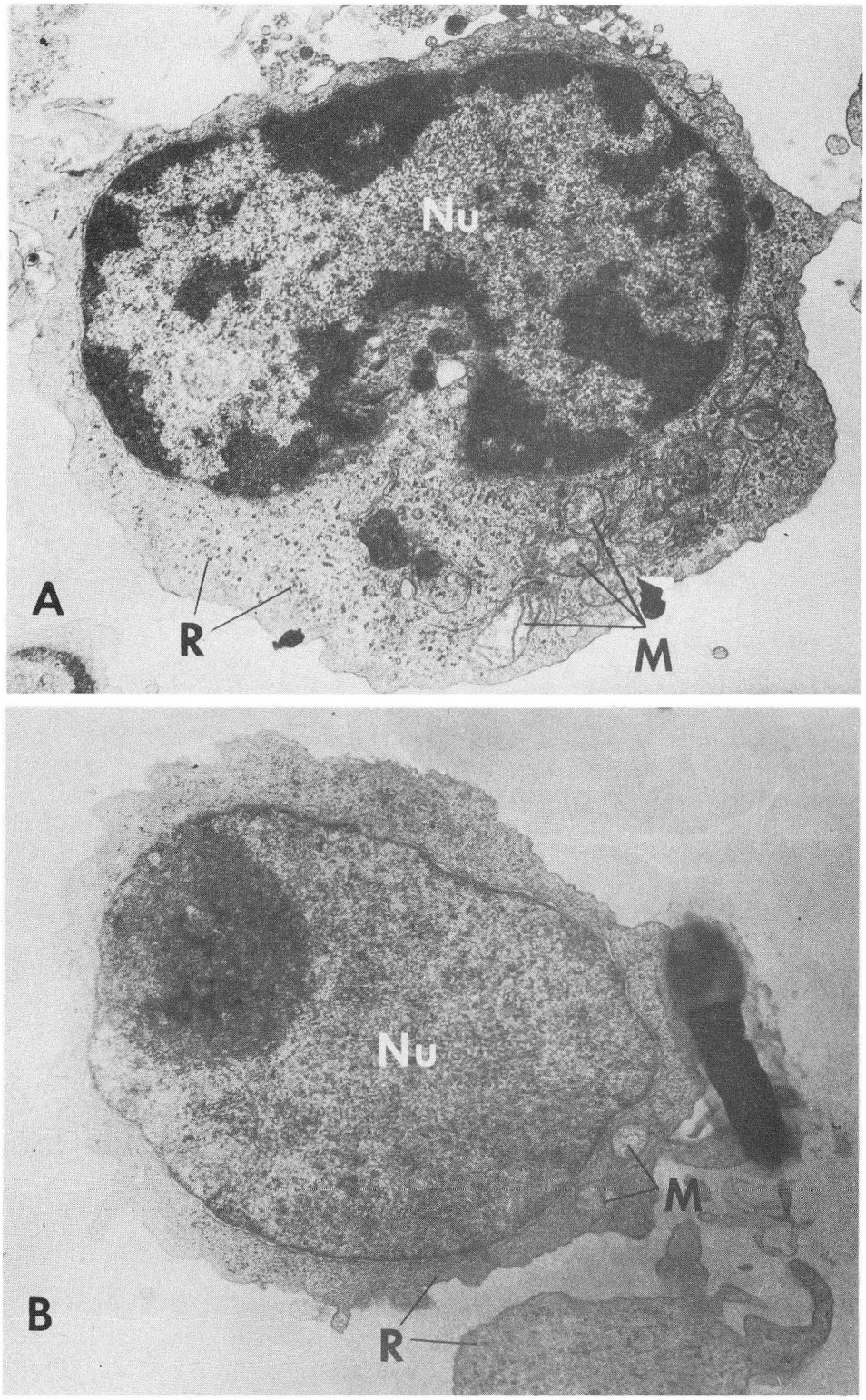
A. Normal cell. There are few mitochondria; but many ribosomes are seen, usually in clusters. Magnification = 18,900x.

B. FA cell. The general appearance is like that of the normal cell. The dark object on the right is a preparative artifact. Magnification = 18,900x.

Nu = nucleus

M = mitochondria

R = ribosomes



XBB 734-2451

Figure 22. Electron micrographs: comparison of cultured lymphocytes from FA and normal individuals.

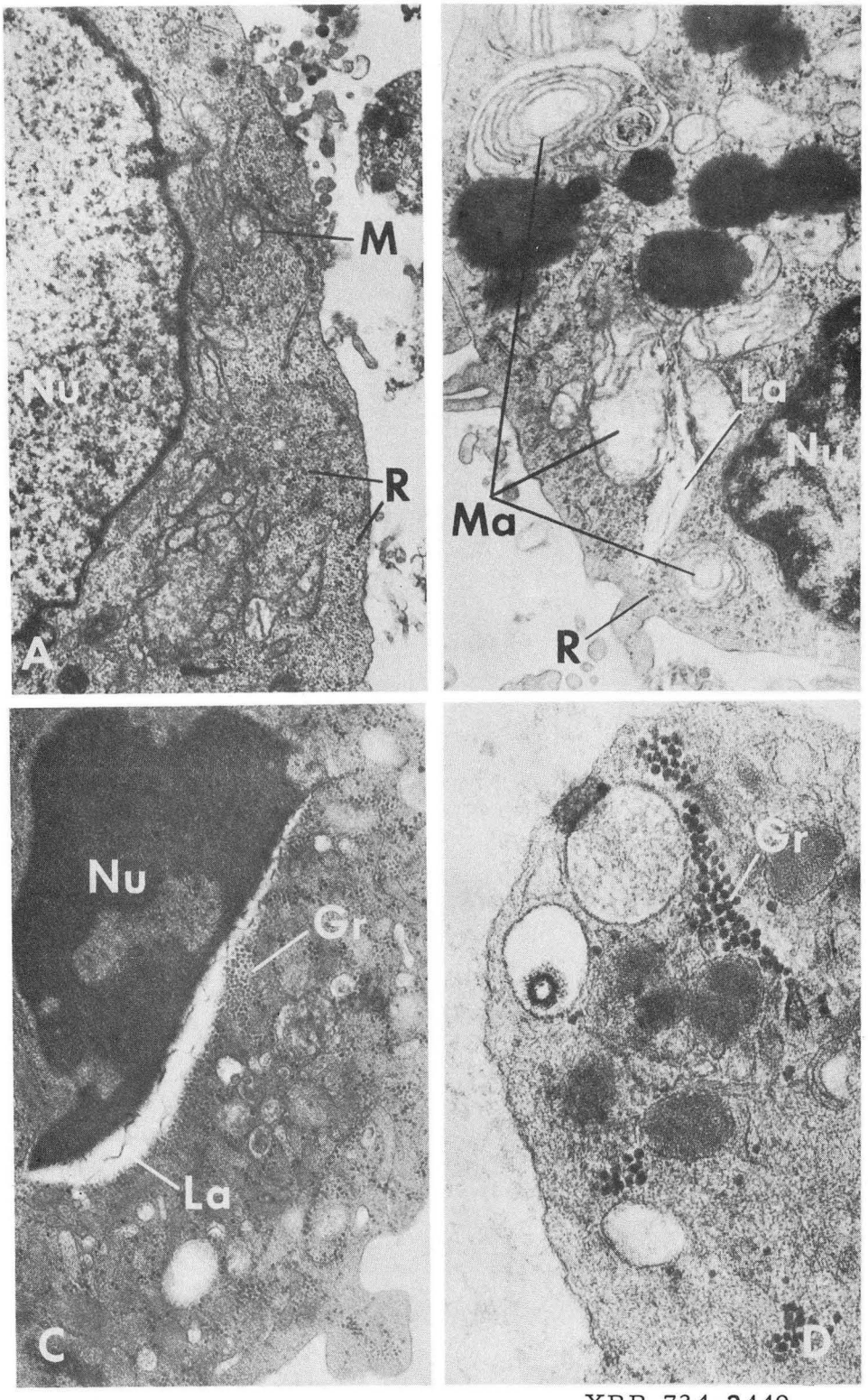
A. Normal cell. Mitochondria are more numerous than in fresh cells. The endoplasmic reticulum is more prominent; there is no marked accumulation of granules as in the FA cells. Magnification = 18,900x.

B. FA cell. As in normal cells, mitochondria are much more numerous than when seen fresh. However many mitochondria are unusually large and have oddly arranged and often concentric cristae. This particular cell has a normal frequency of ribosomal clusters. The large dark areas are thought to be lysosomal bodies. One of the lacunae found in the endoplasmic reticulum is seen. Magnification = 18,900x.

C. FA cell. A characteristic of the cultured cells obtained from this FA case is the odd lacunae found in the endoplasmic reticulum, often as in this cell close to the nucleus. The dense accumulation of small granules thought to be glycogen is apparent. Ribosomal clusters are rare. Magnification = 38,600x.

D. FA cell. This higher magnification micrograph shows the granular clusters seen in (C) very clearly, and further demonstrates the reduction in frequency of ribosomal clusters. Magnification = 58,500x.

Nu = nucleus; M = mitochondria; R = ribosomes; La = lacunae in endoplasmic reticulum; Ma = abnormal mitochondria; Gr = granular clusters.



XBB 734-2449

a marked accumulation of granules presumed to be glycogen, a peculiar defect of the endoplasmic reticulum, and a reduction in numbers of polyribosomes. The mitochondria in the FA cells, like those in the control cultures, are enlarged physically and also reduced in quantity. However the changes in the FA cells appear to be more marked, and the cristae often appear to be concentric. Extrapolating from this 2-dimensional view, the cristae seem to be arranged in layered spherical or cup-shaped groupings on the mitochondrial wall. No other marked changes are apparent: viral or proviral bodies are not seen.

Discussion

The significance of the lacunae in the endoplasmic reticulum, and the reduced numbers of polyribosomes, is not known. Similar lacunae are often found associated with reduction of ribosomes in embryonic and in some neoplastic cells, but never in normal cells [46]. Cells in senescent cultures often have bizarre mitochondria, and the present findings may be an extreme early example of a phenomenon expected to occur later in culture [36]. The accumulation of glycogen granules, and their frequent appearance in bunches, is a most unusual observation, the significance of which is obscure [36,46]. As this suggests a change in the cells' carbohydrate metabolism, there could be

a connection with the increased incidence of diabetes reported among FA relatives [129].

Failure to find viral bodies is not conclusive insofar as any relationship between FA and viruses may be concerned. But failure to find them in lymphocyte cultures is strong suggestive evidence that viruses are not responsible for the increased cell death in FA lymphocyte cultures, nor for the increased chromatid breakage found in the cross-incubation experiments. The latter conclusion lends support to the negative findings of others who have performed cross-incubation experiments [11,55,91]. The increased chromatid breakage in the present cross-incubation experiments must be due to a cellular product accumulated in the FA medium.

Allison, and Allison and Paton [1,2], suggested enzymes released by destruction of lysosomes might produce chromosome breaks in FA cells. However, Cohen et al. and Hittelman [146,147] have indicated loss of lysosomal integrity may be unrelated to chromosome damage. In the present study there did not appear to be any qualitative difference between FA and control lysosomes, and their frequencies appeared to be nearly the same in both cell types. To this degree, there is no evidence of lysosomal involvement in the chromosome aberrations seen in FA lymphocytes.

CHROMOSOME REPAIR TIME DURING G_1 *In Vitro*

The inverted ratio of chromatid exchanges to chromatid breaks has significant implications for the chromosome repair process. These were discussed in an earlier section; experiments to determine the time available for repair have been described. The alternative proposition of alteration in the rate of chromosome repair in FA cells is examined in the experiments described below.

Materials and Methods

In July 1969 and both March and May 1970 PHA-stimulated lymphocyte cultures were established from the FA propositus, her family members, and control subjects, in the manner described before. After 20 hours of culture the samples were irradiated using the "split-dose" techniques used by others in similar experiments [93,94,142,143].

Cultures in flasks were washed by centrifugation in Hank's Balanced Salt Solution (HBSS)[1], transferred with plasma-free fresh medium to stoppered plastic culture tubes incubated in a metal-block heater[2], and sequentially irradiated under a Philips X-ray therapy ma-

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[1]Hank's Balanced Salt Solution. Microbiological Associates, Albany, California.

[2]"Multi-Temp Block". Lab-Line Instruments, Inc., Melrose Park, New Jersey.

chine[1] operated at 250 kvp, 15 ma, with 5 mm Al filtration. Plasma-free medium was used to avoid possible artifact due to irradiation of plasma [118]. A 20 inch culture-to-tube distance reduced beam cross-sectional flux variation to 1% between cultures. A Victoreen condensor type R-meter[2] was used to measure exposure rates of 1 R/sec inside empty plastic culture tubes when total exposure was controlled by the therapy machine's interval timer. Use of plastic rather than glass culture tubes during irradiation served to minimize dose build up from secondary electrons at the tube-medium interface [31, p.576]. Cultures were not incubated during the 2 1/2 minute duration of X-ray exposure.

Starting at 20 hours, the cultures from each subject were exposed to 268R or 300R of X-rays, with one culture each reserved as an unirradiated control. The exposures were delivered in two equal portions, separated by 0, 1/2, 1, ... etc. hours, to a maximum of 5 1/2 hours interval between exposures. Following each irradiation the cultures were washed by centrifugation and replenished with complete medium plus PHA.

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[1]Therapy Apparatus Type 11645. N.V. Philips' Gloeilampenfabriken, X-Ray and Medical Apparatus Division, Eindhoven, Holland.

[2]The Victoreen Instrument Company, Cleveland, Ohio. This particular instrument had been calibrated 9 months previously by the U.S. Bureau of Standards.

Incubation was then continued in the usual humid CO_2 atmosphere. At 68 hours of culture Velban was added for 1 hour and the cultures were harvested.

Air-dried or LPO squash chromosome preparations were made as described before, and at least 100 cells per datum were examined for each subject. Cells were examined at 1250-1600 diameters magnification, and the number of dicentric, multicentric, and ring chromosomes found was recorded. The average fraction of cells with dicentrics and rings was calculated, along with the 95% confidence intervals for the observations. The resulting data were plotted as the percentage of cells with dicentrics and rings as a function of the time interval between half-exposures. Differences between the resulting curves were assessed for statistical significance by conventional methods [123,125].

The constant of proportionality between aberration yield and exposure was computed, assuming the relation

$$Y = c + aD^2 \qquad [31,p.415;32]$$

where Y = observed yield of dicentrics and rings per cell,

c = the control frequency of dicentrics and rings per cell,

a = the proportionality constant in terms of (dicentrics + rings)/cell/R^2,

and D = the total exposure in R.

The curves for all subjects but the propositus were not significantly different, and their data were

pooled. Aberration yields were obtained by averaging the first and last four data points for the non-FA subjects, and the first and last three data points of the FA case. Yields for the closely-spaced irradiations were considered the yields for the total exposure; yields for the widely-spaced irradiations were considered to be the sums of the separate irradiation yields [143]. Proportionality constants obtained from the 300R irradiation series were averaged with those obtained from the 268R series.

Results

Unirradiated control cultures for all subjects had a frequency of dicentrics and rings below 1%; this was not subtracted from the experimental data shown in Figures 23 and 24. In all but the cultures from the propositus, the frequency of dicentrics and rings was fairly constant for exposure separations of a few hours, then it dropped rather sharply to a much lower and also relatively constant level. The time interval between exposures required to achieve one-half the final reduction in dicentric and ring frequency was taken as the time required for radiation-induced chromosome breaks to restitute. This time interval was very close to 4 hours for all subjects.

The curve for the FA propositus was markedly and significantly different from those from all other sub-

Figure 23. Frequency of dicentrics and rings as a function of time between fractionated X-ray exposures.

Except in the FA case, the frequency of X-ray induced dicentrics and rings is almost constant even for exposure separations of several hours. For greater exposure separations the frequency drops sharply to a new lower almost constant level. In the FA case, the decline in aberration frequency starts with the shortest exposure separation and continues in a nearly linear manner to separations of 6 hours, the maximum used.

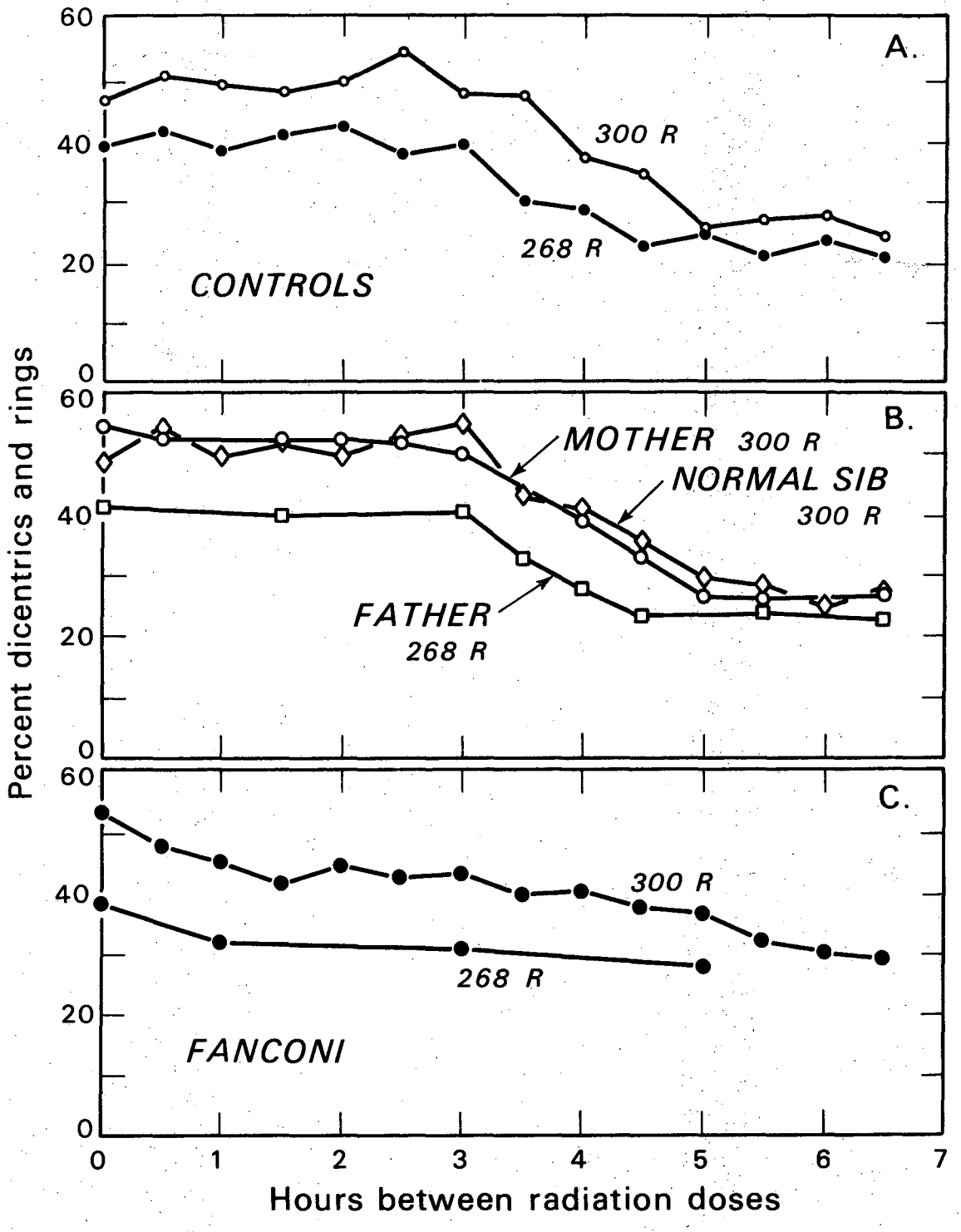
A.
300 R
268 R
CONTROLS
B.
MOTHER 300 R
NORMAL SIB
300 R
FATHER
268 R
C.
300 R
268 R
FANCONI
Percent dicentrics and rings
Hours between radiation doses
0
20
40
60
0
1
2
3
4
5
6
7

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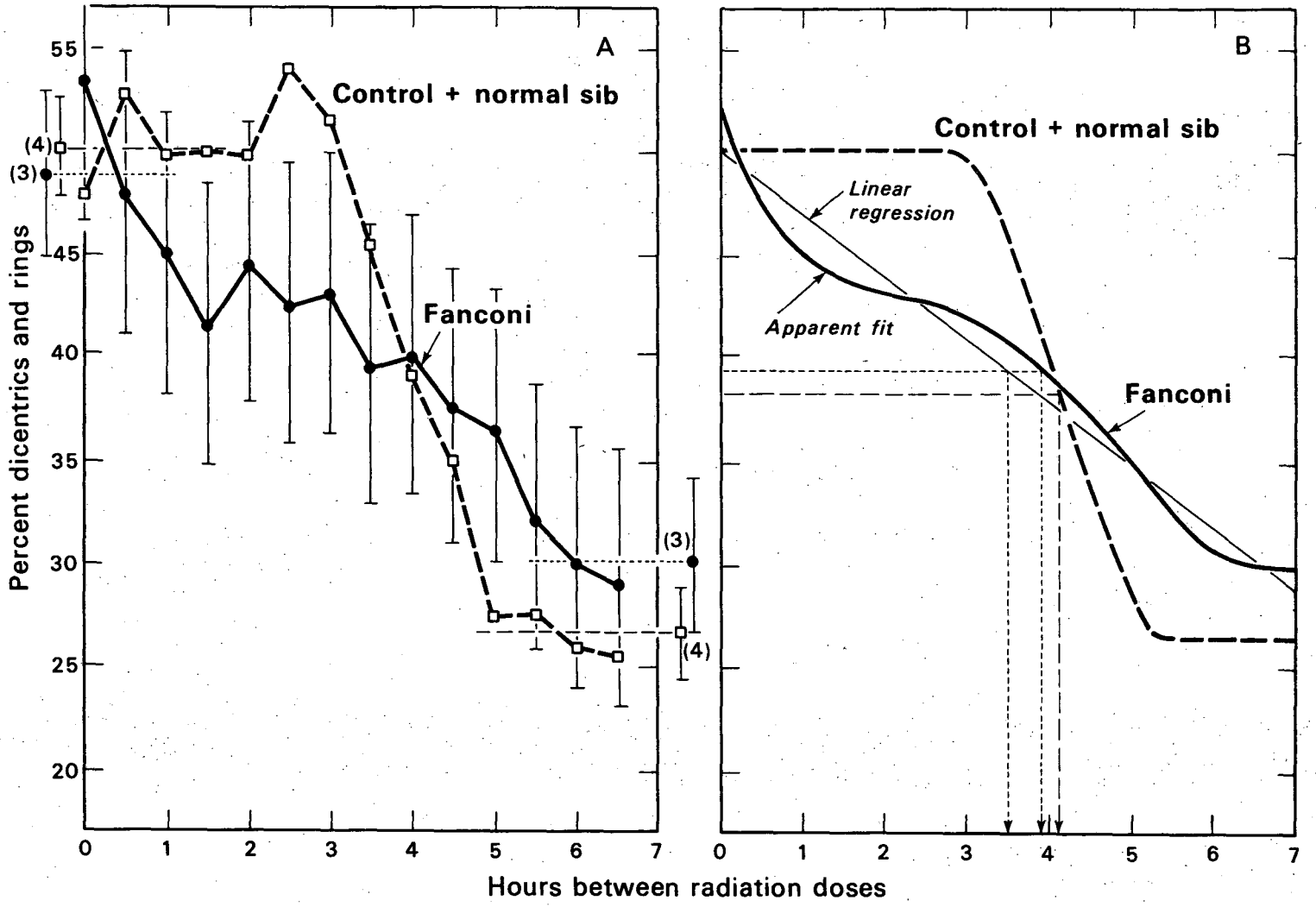
Figure 24. Chromosome repair time during G_1 in cultured lymphocytes.

A. Some data for 300R from Figure 23 are redrawn. The control and normal sib data are combined to yield a composite normal curve. The FA data are redrawn with 95% confidence intervals on each datum. The FA curve is significantly different from the normal curve.

B. The curves in A are smoothed and redrawn. The FA curve is not significantly different from a straight line, but it might also show a sharp initial drop followed by a curve similar to the normal curve. The mean chromosome repair time is 3 1/2-4 hours in both normal and FA subjects.


A
B
Control + normal sib
Fanconi
Linear regression
Apparent fit
(4)
(3)
Percent dicentrics and rings
Hours between radiation doses
55
45
40
35
30
25
20
0
1
2
3
4
5
6
7

DBL 707-5815

jects. It showed an initial dicentric and ring frequency similar to that of other subjects, followed by a steady decline with time interval between exposures. The initial plateau followed by a sharp drop to a lower plateau characterizing the other subjects' curves was not seen. The curve may be interpreted as a linear reduction in dicentric and ring frequency with increasing time interval, or as a complex curve including a sharp initial drop with a central plateau, and subsequent final drop. In either case the mean chromosome break restitution time is very close to that found in the other subjects.

Aberration yield proportionality constants as a function of exposure for the FA propositus and for the other subjects combined are shown in Table 10. The yields for the FA propositus are lower than for others at small exposure separations, but higher at large separations.

Discussion

The 4-hour break restitution time shown by all subjects is much longer than the 90 minutes given by Prempree and Merz [93,94]. However they irradiated cells which had been in culture for 48 hours, when they believed most cells were in S-phase, and they scored chromatid exchanges immediately after the conclusion of their experimental schedule. Apart from probable

Table 10. Proportionality constants for yields of radiation-induced chromosome aberrations in cultured lymphocytes from study family members

	Aberration yield: (Dicentrics + rings) x 10^{-6}/cell/R^2	
	(300R^2) plateau	(2 x 150R^2) plateau
Control (300R)	5.4	5.8
Control (268R)	5.6	6.2
Control average	5.8 x 10^{-6}/cell/R^2	
Father (268R)	5.6	6.4
Mother (300R)	5.9	5.9
Normal sib (300R)	5.7	6.1
Family average	5.9 x 10^{-6}/cell/R^2	
FA sib (300R)	5.4	6.7
FA sib (268R)	4.9[1]	(7.7)[2]
FA average	5.7 x 10^{-6}/cell/R^2	

[1]Calculated from first two data points.

[2]Single point, possibly not on plateau, not used in average for FA.

inaccuracies in their assumptions, their data are not so easily interpreted as they suppose. In any event, their result is based on limited data and, if accurate, pertains to repair in S or G_2. The 4-hour interval determined here plainly is the G_1 chromosome repair time, and it corresponds closely to the time found by Wolff [142].

While the chromosome repair time determined for the FA propositus is the same or slightly less than that for the other subjects, the curve used to derive the value is aberrant. If her curve may be interpreted as a straight line, it is possible to consider the curve as resulting from repair times randomly varying throughout the cell population. Some repair times would be very short, some quite long, with an average value fortuitously close to normal. Alternatively, two populations of cells with quite different but still discrete repair times could give a complex curve. The initial sharp drop in aberration frequency followed by a plateau suggests a population with very short repair times, or one markedly resistant to radiation-induced chromosome damage. The subsequent drop from the central plateau suggests a population with normal or longer than normal repair times.

Rather than showing either a single time-varying process, or multiple cell populations, the FA curve could be interpreted equally well as evidence for two

distinct repair processes in the FA cells. In some similar experiments on Vicia faba, Wolff and Luippold found an initial sharp drop in aberration frequency [144]. They interpreted this in terms of a very rapid restitution of breaks in ionic bonds. No similar initial drop is seen in the other subjects' cells. It is possible that FA chromosomes are more susceptible to ionic bond breakage than are normal chromosomes. Alternatively the normally very rapid restitution of such breaks may be somewhat slowed in FA cells. A variety of explanations of the unusual FA curve may be given; they remain completely speculative. However it does appear that some break repair in the FA cells is more rapid than normal (a high repair rate), and some is slower than normal (a low repair rate).

The coefficients of proportionality between yield of dicentrics and rings and the square of the exposure, agree well with those given by Bender and Gooch [8,9]. However they are about twice those given by some [32,33,80,81] and half or less those given by others [52,106]. Differences from other work may arise from assuming an exposure exponent equal to 2 when it may be rather less than that [32,119], by assuming negligibility of all formula terms other than that given here [9,32], or through use of different times of irradiation and sampling [32,52]. In any event the proportionality constants are within the

range of those found by others using human leukocytes, and they are consistent among the subjects used here.

The proportionality constants obtained from the propositus are both lower (for small time intervals) and higher (for long time intervals) than for the other subjects. From the nature of the curve, it seems likely these differences result from averaging aberration yields which are not, in fact, similar. At both first and last data points the aberration yields are close to those found in the other subjects. It appears that the true proportionality constant may be close to that of the other subjects.

Higurashi and Conen found a four-fold increase in chromosome aberrations in irradiated FA cells [54]. The present data are restricted to dicentrics and rings, but to that extent do not support their results. These workers irradiated cells in medium containing plasma. Scott showed that irradiated plasma might induce chromosome breaks in cultured lymphocytes [118], and this effect may have contributed to the results of Higurashi and Conen. Alternatively, FA patients may differ in the radiosensitivity of their chromosomes.

SENSITIVITY TO CHROMOSOME BREAKAGE DURING G_2 *In Vitro*

The possibility of some insensitivity of FA chromosomes in G_1 to radiation-induced breakage was raised earlier. Higurashi and Conen, on the other hand, showed FA chromosomes were about 4-fold more sensitive to radiation-induced breakage than were control cells [54]. In both instances, the event observed was related to the cells' first G_1 in culture [32]. Left open was the question of sensitivity to breakage during S or G_2, an issue more pertinent to the origin of the chromatid exchanges seen in FA cases. An experiment was performed to determine the sensitivity of FA cells to radiation-induced chromosome breakage during G_2.

Materials and Methods

Lymphocyte cultures were established in March 1969 as described before, using cells from the FA propositus and one normal individual. These were cultured for 65 hours in the presence of PHA, then washed by centrifugation in HBSS, placed in plastic culture tubes with HBSS, and exposed as described before to either 50R or 150R of X-rays. Following exposure the cells were centrifuged, the HBSS was discarded, and the cultures were replenished with complete medium. After an additional 5 hours of incubation, including exposure to Velban during the terminal hour, the cultures were

harvested and chromosome preparations were made as described before.

Slides were scanned under low power (125 diameters magnification), and 100 or more selected well-spread metaphase cells from each subject were examined at 1250-1600 diameters magnification for each datum. Chromosome aberrations were scored as before, with special attention given to the presence of chromatid exchanges.

Results

With the exception of chromatid exchanges and dicentrics found as expected in the FA cells, virtually all the radiation-induced chromosome aberrations were simple chromatid breaks. The ratio of breaks found at the two exposure levels is close to 3:1 in all subjects. The FA cells contain a higher frequency of chromatid breaks than do control cells at all exposure levels, but the difference is not significant. Irradiated FA cells do not contain a higher frequency of chromatid exchanges than do unirradiated FA cells. The data are summarized in Table 11.

Discussion

Although 4 hours was determined to be the G_1 chromosome repair time for normal cells, there was no discernible tendency of any subject's cells to have an increased frequency of chromatid exchanges 5 hours

Table 11. Frequencies of chromosome aberrations induced by radiation during G_2 in cultured lymphocytes of the FA propositus.[1]

		CHROMATID ABERRATIONS										CHROMOSOME ABERRATIONS						
		BREAKS		CHROMATID EXCHANGE C/C						ISO/CD								
				INTERCHANGE		INTRACHANGE										DELETIONS		
						INTER-ARM		INTRA-ARM							AC			
		CTD	ISO	SYM	ASYM	SYM	ASYM	SYM	ASYM	INTER	INTRA	DIC	RING	TRI	R	I.D.	TERM	GAPS
0 R	CONTROL																	3
	FA	7		8	6					1		2						5
50 R	CONTROL[3]	14	1															10
	FA[3]	27	1	7	10							4					1[2]	7
150 R	CONTROL[4]	47	12															19
	FA[4]	46	10	6	7					1		2						15

[1] *Tabulated values are % of cells (100 cells observed per subject per exposure)*

[2] *No associated fragment; presumed to be aberration in prior division*

[3] *After subtracting '0' R value: Control = 0.0030 bks/cell/R ; FA = 0.0042 bks/cell/R*

[4] *After subtracting '0' R value: Control = 0.0039 bks/cell/R; FA = 0.0033 bks/cell/R*

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after irradiation. This suggests either the chromosome repair time in G_2 is different from that in G_1, or that misrepair did not occur in G_2 to an appreciable degree. The former contention receives some support from the work of Brooks and Lengeman using testes and marrow of the Chinese hamster [16]. They found average times that chromatid lesions remained scorable were approximately 6 and 7 hours, respectively.

The latter conclusion seems startling, as chromatid exchanges are widely held to be created in either or both of S and G_2. It is possible however, that radiation may have induced a lag in cellular development similar to that found in the cell cycle experiments. In this event, severely damaged cells might be more laggard than others, and would not be seen in metaphase 5 hours after the injury. Thus it is possible that chromosome repair and misrepair would have occurred at a time after the termination of the experiment.

Selection of one of these alternatives over the others is not possible, but on the data's face, FA chromosomes are not shown to be more radiosensitive during G_2 than are normal chromosomes.

In passing, it may be noted that the dose-response relations for chromatid breaks and chromatid gaps are concave upward at increasing radiation exposure. This response would be expected on Revell's exchange hypoth-

esis, although the data are also consistent with a strictly linear relationship of aberrations to exposure.

THE TIME SEQUENCE OF CHROMOSOME ABERRATIONS *In Vitro*

The FA case described in this report has a high frequency of chromosome aberrations in cultured lymphocytes, and few or none in fresh or cultured marrow cells. Most previous reports dealing with marrow cells from FA cases cite either a low frequency or total absence of chromosome aberrations in the marrow [11,109,128]. If marrow cells had been subjected to the same injurious process resulting in lymphocyte chromosome aberrations, then it would be expected that the resulting cell lethality could explain both the anemia and the low aberration frequency. Cells with aberrations would die more commonly than normal cells, and normal cells would predominate in rapidly dividing tissues like marrow. Lymphocytes, with their long circulating lifetime [17,80,81,82,83] could accumulate injury which would be revealed as a high frequency of aberrations during the first divisions *in vitro*.

This hypothesis suggests that chromosome aberrations in lymphocytes *in vitro* should decline in frequency with time in culture after divisions first occur. An experiment was performed to test this prediction.

Materials and Methods

Lymphocytes were obtained from the FA propositus and established in PHA-stimulated cultures in March

1970, in the manner previously described. After hypotonic treatment and fixation, chromosome preparations were made as LPO squashes from small aliquots of a single culture. The frequencies of simple breaks, chromatid exchanges, multicentric chromosomes, and endoreduplicated cells were determined four times at intervals of 24 hours, starting at 48 hours. The aberration frequencies were plotted as functions of time in culture. When appropriate, regression relations were obtained by standard methods, and 95% confidence intervals for the observations were calculated.

Results

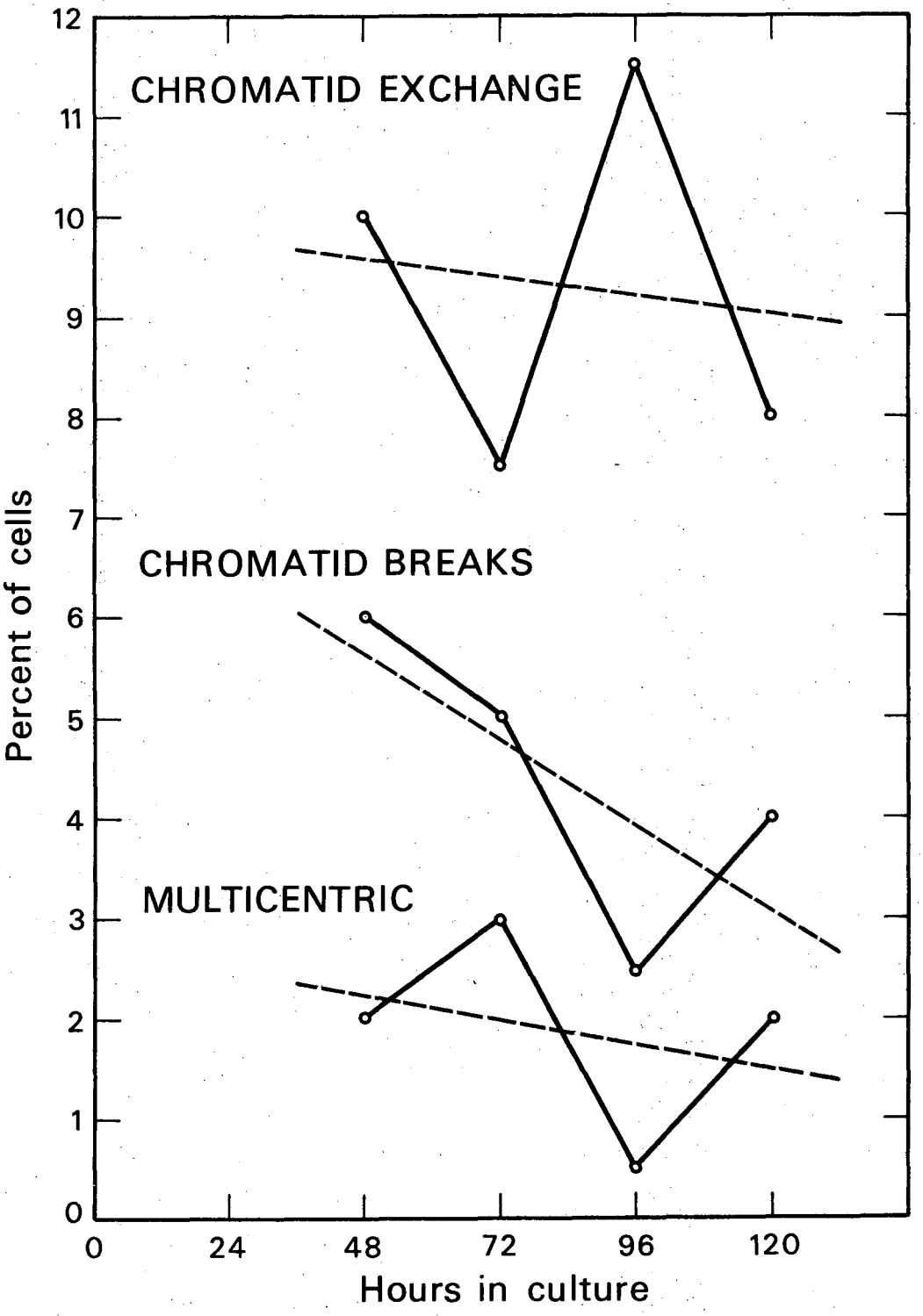
The time-dependent frequencies *in vitro* of the several types of chromosome aberration are shown in Figure 25. The data do not produce elegant curves, but it is evident that the aberrations did not disappear with time in culture, nor did their frequency markedly decline during the 3 day observation period.

Discussion

As hypothesized, it is reasonable to believe that gross chromosome abnormalities contribute to observed cell lethality in populations of dividing cells. This would be particularly true of di- and multicentric chromosomes, a proportion of which are expected to produce bridges during cell division, followed by breakage and genetic imbalance in the progeny cells. Loss

Figure 25. Chromosome aberration frequencies in cultured FA lymphocytes as a function of time in culture.

Dashed lines are regression lines for the data shown. All classes of aberration show only moderate declines in frequency from the second through the fifth day of culture. The moderate decline of aberration frequency does not accord with the high rate of cell death seen in the FA cultures, unless aberrations tend to be created de novo in each cell generation.


CHROMATID EXCHANGE
CHROMATID BREAKS
MULTICENTRIC
Percent of cells
Hours in culture
0
1
2
3
4
5
6
7
8
9
10
11
12
0
24
48
72
96
120

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of these cells would reduce the frequency with which dicentrics are found. Yet the dicentrics do not decline significantly in frequency, suggesting they are created constantly at a rate nearly equal to their (expected) loss. Chromosome-type abnormalities in general are uncommon, and the chromatid exchange frequency hardly declines. The observations strengthen the hypothesis that multicentrics are derivatives of chromatid exchanges after cell division.

The frequency of chromatid exchanges is approximately constant, and exceeds the slightly declining frequency of simple breaks. As before, both must be created de novo in each cell generation in culture. Since the cells in culture are not exposed to radiation or chromosome-damaging agents, the de novo creation must be due to a condition within the cells themselves.

THE TIME SEQUENCE OF CHROMOSOME ABERRATIONS *In Vivo*

Since Schroeder's initial investigations [115], a rather large number of cytogenetic investigations on cases of FA have been reported. Virtually all agree that aberrations in cultured FA lymphocytes are much more frequent than in normal individuals' cells; they do not agree well on details. Most studies have revealed a greatly increased frequency of chromatid exchanges, but some authors report none at all [55]. Endoreduplication is a common finding in some studies [e.g. 55], but rare or absent in others [e.g. 108]. The situation is complicated by several uncontrolled variables: The patients cited in prior studies had been receiving various medications for varying lengths of time, or had received none at all. Additionally, the studies were performed at varying times after the diagnosis of FA.

The case of FA reported here was first studied cytogenetically at the time of diagnosis, before any medications had been administered, and only a few weeks after the first symptoms had been noticed. To help resolve some of the confusion surrounding the cytogenetic findings in FA, a prospective study of the time course of chromosome aberrations in FA was begun.

Materials and Methods

Lymphocytes from the FA propositus were obtained

and cultured as previously described on 7 occasions: three times in 1969, and once each in 1967, 1970, 1972 and 1973. Marrow samples were obtained and prepared as previously described on four occasions: once each in 1967, 1968, 1969 and 1972. Cytogenetic analyses were made as before on at least 200 cells for each date. The frequencies of chromatid exchanges, chromatid breaks, and dicentrics plus rings were graphed as functions of time since diagnosis. The 95% confidence intervals for the observations, and regression relations when appropriate, were calculated as before.

Results

Cytogenetic observations made on lymphocytes and marrow cells of the FA propositus are tabulated in Table 12. The frequencies of major classes of chromosome aberrations in lymphocytes are plotted in Figure 26 as a function of time after diagnosis. The data for chromatid exchanges in Figure 26 are repeated in Figure 27, with the regression line fitting the data also shown.

The aberration frequency in marrow cells is unremarkable on all four observation dates. The micronucleated cells seen in the initial study were found in every sample.

In lymphocytes, all true aberration classes decline in frequency with time, except endoreduplica-

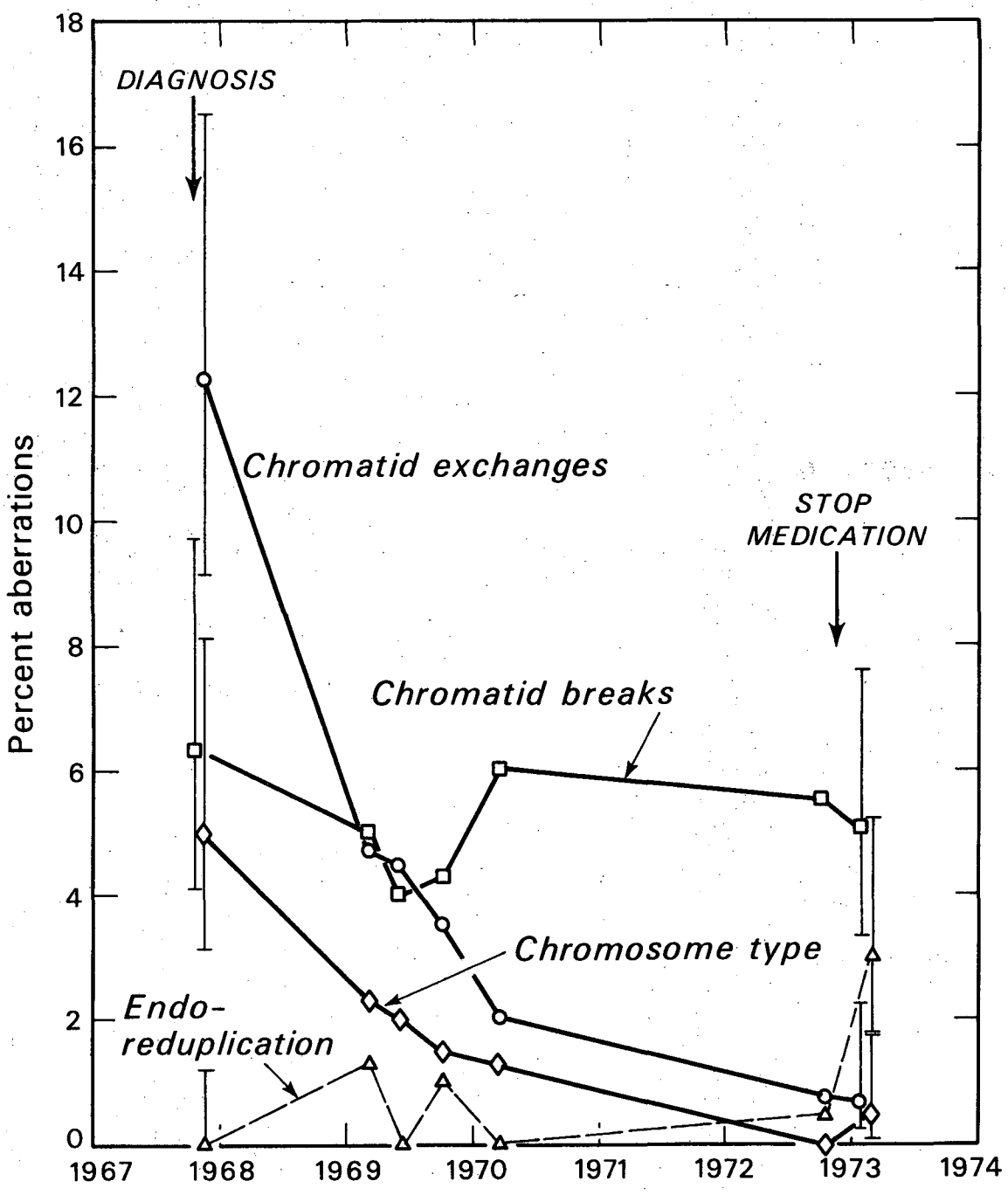
Table 12. Chromosome aberration frequencies during the course of FA: Cultured lymphocytes and marrow cells.

DATE	CHROMATID ABERRATIONS										CHROMOSOME ABERRATIONS							ENDO-REDUPLI-CATION	NO. CELLS
	BREAKS		CHROMATID EXCHANGE C/C						ISO/CD						DELETIONS				
			INTERCHANGE		INTRACHANGE														
					INTER-ARM		INTRA-ARM												
	CTD	ISO	SYM	ASYM	SYM	ASYM	SYM	ASYM	INTER	INTRA	DIC	RING	TRI	AC R	I.D.	TERM	GAPS		
LYMPHOCYTES																			
13 NOV 1967	16	4	15	13		1			8		13		2				25		300
5 MAR 1969	12	3	6	5					3		6		1				19	4	300
22 JULY 1969	8		5	2					2		4						15		200
4 AUG 1969	7	2	3	3					1		3						18	2	200
11 FEB 1970	17	1	4						2		2						24		300
10 OCT 1972	14		2	1													29	2	400
24 JAN 1973	18	2	1	1					1		2						22	12	400
MARROW CELLS																			
25 OCT 1967	1																3		200
27 SEPT 1968	2																1		100
22 JULY 1969	1															1			100
11 OCT 1972	1																1		100

DBL 734-5123

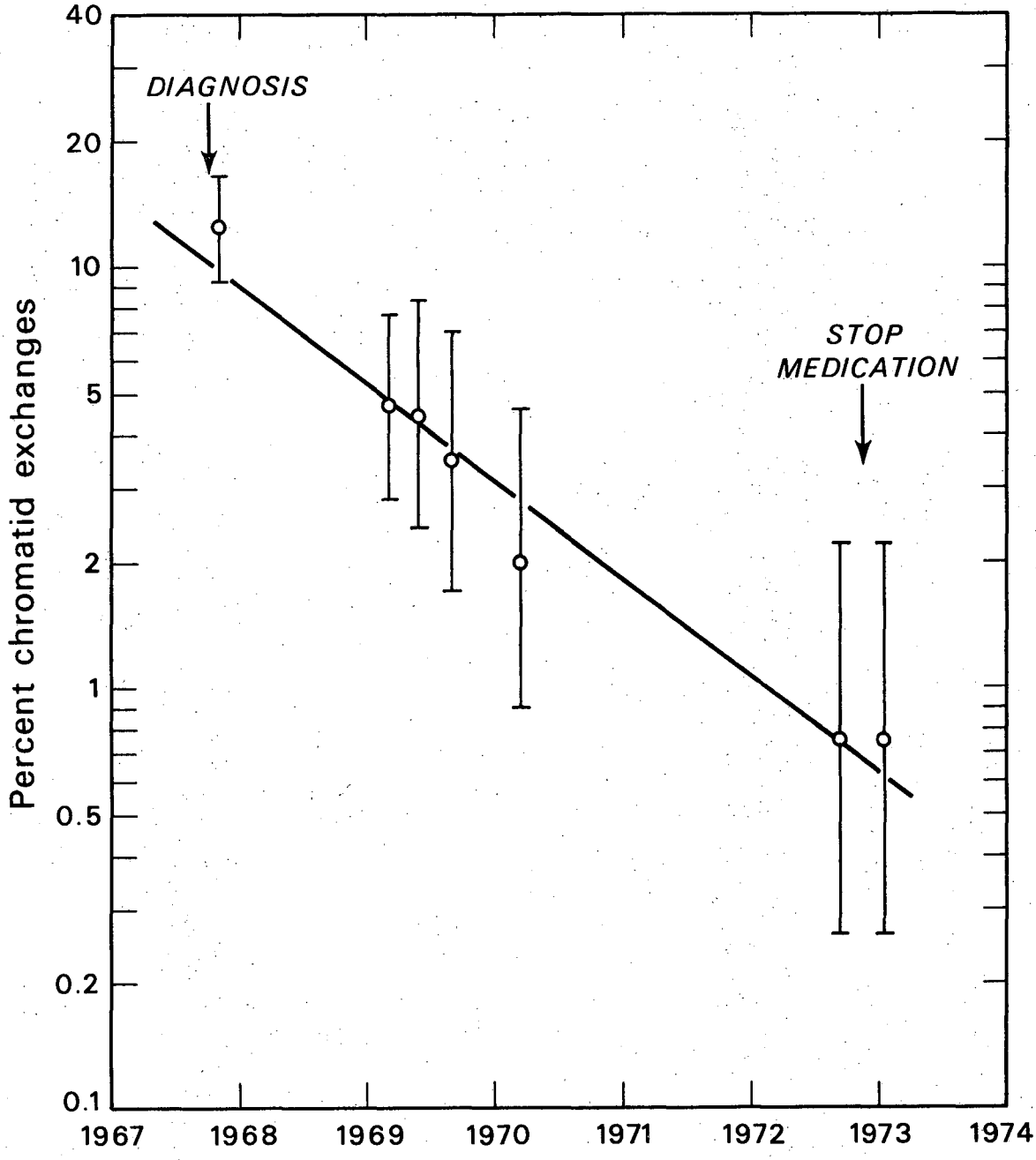
Figure 26. Chromosome aberration frequencies found in cultured lymphocytes of the FA propositus during the course of the disease.

Between 200 and 400 cells were observed on each date to obtain the frequencies shown. Vertical lines are 95% confidence limits for the percentages. Chromatid exchange and chromosome-type aberration frequencies decline markedly with time; chromatid break frequencies are relatively constant. Endoreduplication is present but relatively uncommon through five years from the time of diagnosis; its frequency rises sharply after stopping treatment. The ratio of chromatid breaks to chromatid exchanges declines with time, and inverts by two years after diagnosis and treatment.


DIAGNOSIS
Chromatid exchanges
STOP
MEDICATION
Chromatid breaks
Chromosome type
Endo-
reduplication
Percent aberrations
0
2
4
6
8
10
12
14
16
18
1967
1968
1969
1970
1971
1972
1973
1974

DBL 734-5113

Figure 27. The regression on time of chromatid interchange frequencies in cultured FA lymphocytes during the course of the disease. The chromatid interchange frequencies of Figure 26 have been redrawn. Vertical lines are 95% confidence intervals for the percentages. The decline in chromatid interchange frequency is simply exponential, with a half-time very like the half-life of lymphocytes *in vivo* (see text).


40
20
10
5
2
1
0.5
0.2
0.1
Percent chromatid exchanges
DIAGNOSIS
STOP
MEDICATION
1967
1968
1969
1970
1971
1972
1973
1974

DBL 734-5114

tion. This was absent shortly after diagnosis, was infrequent in later years, but its frequency rises sharply in 1972 - after all medication ceased.

The decline in chromatid exchange frequency over time appears to be exponential with a half-time ($T_{1/2}$) of 16 months. The mean time of disappearance (mean time = $T_{1/2}/\ln 2$) is 23 months.

The disappearance rates of the other aberration classes appear to be inconstant. Dicentrics and multicentrics are always less frequent than chromatid exchanges. Simple chromatid breaks, initially significantly less frequent than exchanges ($t = 2.65; p < 0.01$), exceed the exchanges by 1972. At that time the excess of breaks over exchanges is significant ($t = 3.62$; $p < 0.001$). Chromatid and isochromatid gaps vary with time in an inconstant manner but do not appear to decline with time.

Discussion

The seven lymphocyte analysis dates span the time between diagnosis and the immediate present. The initial study was performed prior to administration of any medications, and the last one three months after medication was discontinued. Of the remaining five studies performed while medication was prescribed, one preceded remission by a month or so, and one was done within four months after remission was observed.

The lack of chromosome aberrations in the marrow accords with some previous observations [11,109], but not with others [128]. However the aberrations found by others in the marrow of an FA patient were only moderately frequent. It seems likely that chromosome aberration in FA marrow cells is an infrequent and uncharacteristic phenomenon.

As discussed in an earlier section, finding virtually no aberrations in lymphocytes except simple breaks, chromatid interchanges, and dicentrics, was an informative observation. In general the same observations are made throughout the patient's course. Dicentric and multicentric chromosomes are never more frequent than exchanges, and decline in frequency with time in a manner similar to exchanges. This is consistent with the hypothesis discussed earlier that dicentrics in FA arise from chromatid interchanges as a result of cell division.

Simple chromatid breaks are more frequent than exchanges early in the course of the disease, but become less frequent later on. Thus the initially inverted break:exchange ratio becomes more normal with the passage of time. This reversion is almost completely due to the steady disappearance of chromatid exchanges with a half-time of disappearance of about 16 months or a mean time of disappearance of 23 months.

Norman [80], Norman and Sasaki [81], and Norman

et al [82,83] have suggested an 18 ± 2 months lifetime for lymphocytes *in vivo*. Buckton *et al.* [17] have estimated their lifetime at 29 months, and Norman et al [82] feel this is an upper limit. The 23 month mean life of the lymphocyte chromatid exchanges reported here is intermediate between those two values. It is easy to believe that lymphocytes "at risk" for chromatid exchange production survive a normal lifespan, and are steadily replaced by lymphocytes not subject to such risk. Since the decline in exchange frequency begins coincidently with the start of therapy, it is tempting to suppose that some cell injury process was halted by the medication.

Cessation of the medication has not been accompanied by an increase in chromatid exchanges, but endoreduplication has become much more frequent. The significance of this change cannot be assessed at this writing.

CYTOGENETIC OBSERVATIONS ON OTHER FAMILY MEMBERS

Cytogenetic aberrations are found regularly in cultured lymphocytes of FA cases (e.g. 112-116), but no similar findings in parents' cells have been reported. Some non-anemic relatives are said to display chromosome abnormalities [55,91]. It is not known whether cytogenetic changes in FA are a late consequence of the condition, as the anemia is often, or a phenomenon which precedes clinical symptoms. By all the usual clinical criteria the FA propositus reported here was quite normal at birth and for 9 years until anemia was detected. The family contains one sibling, normal at birth, who remains clinically normal to this date. Demonstration of, or failure to demonstrate, cytogenetic changes in the normal members of FA families would have important consequences for theories of FA etiology. Therefore cytogenetic analyses were performed on the parents and normal sib of the present FA case.

Materials and Methods

Peripheral blood was obtained from the parents and normal sib of the FA propositus during June 1968 and March 1971. Lymphocyte cultures were established and cytogenetic analyses were performed as described earlier.

Results

The results of cytogenetic analyses on the mother,

father, and normal sib of the FA propositus are shown in Tables 13 and 14. All three subjects are normal with respect to the distribution of numbers of chromosomes per cell and frequencies of chromosome aberrations.

Discussion

The absence of cytogenetic aberrations in the cultured lymphocytes of the parents and the normal sib confirms the observations of others (see 11, 41, 108 and 116 for reviews).

These analyses and other (normal) experimental results reported here constitute a baseline against which any future clinical changes may be measured. Should the normal sib eventually display clinical or cytogenetic features of FA, changes in the observed parameters would help define FA's etiology. Periodic restudy is indicated, and further analyses are planned.

Table 13. Distribution of numbers of chromosomes per cell in cultured lymphocytes from other family members.[1,2]

	Number of chromosomes per cell											
	≤40	41	42	43	44	45	46	47	≥48	~4n	>4n	Total cells
Father 1968				1		1	98					100
1971	4	2	4	2	8	8	72					100
Mother 1968	2		3		4	7	84					100
1971		1			6	9	83		1			100
Normal sib 1968	2	1		1	2	4	89			1		100
1971	1	1		1		2	95					100

[1]Tabulated values are percent of cells observed.

[2]Increased hypomodal values probably are technical artifact.

Table 14. Frequencies of chromosome aberrations in cultured lymphocytes from other family members.

	CHROMATID ABERRATIONS										CHROMOSOME ABERRATIONS						
	BREAKS		CHROMATID EXCHANGE C/C						ISO/CD								
			INTERCHANGE		INTRACHANGE				INTER	INTRA					DELETIONS		
					INTER-ARM		INTRA-ARM							AC			
	CTD	ISO	SYM	ASYM	SYM	ASYM	SYM	ASYM			DIC	RING	TRI	R	I.D.	TERM	GAPS
FATHER																	
1968	1																2
1971											1						5
MOTHER																	
1968	1															1	4
1971	1																8
NORMAL SIB																	
1968	3																13 [1]
1971	1																5

[1] *Values this high are observed on occasion as transient elevations in normal individuals*

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DISCUSSION, AND SOME CONCLUSIONS

Three fundamentally different theories have been advanced to explain the chromosome aberrations found in cultured FA lymphocytes: somatic crossing-over [39, 43], exchange initiation (this report), and breakage-and-reunion (most other authors). Initial cytogenetic observations on the FA case presented here included moderate elevations of the frequencies of both simple chromatid breaks and dicentric chromosomes, greatly elevated frequencies of chromatid interchanges, virtual absence of other aberration types, and an inversion of the expected ratio of breaks to exchanges (Table 3). From a consideration of these findings, the exchange theory was accepted as a working hypothesis subject to test and verification. At the outset it was realized that direct tests of the hypothesis would be difficult in the available material. It was felt that the alternate hypotheses could be tested for their sufficiency in explaining experimental observations. Their failure to explain or to permit the data would be evidence of inapplicability of the hypotheses, allowing provisional consideration of the exchange model.

The somatic crossing-over theory is supported by the increase in chromatid interchanges, and the absence of aberrations other than dicentrics and simple breaks. Dicentrics are permissible under this model,

as they can be derived from the chromatid interchanges [118]. Additionally, homologous chromosomes are more frequently involved in interchanges than non-homologs (Table 4). But non-homologous association is common enough; the theory would have to be stretched to include non-homologous crossing-over. The relative constancy of exchange (and other aberration) frequencies through time in culture (Figure 25) is not inconsistent with the model. But the elevated frequency of simple chromatid breaks at all times is not explained; a second aberration mechanism would have to be proposed. While much of the data obtained both initially and subsequently are explicable by the crossing-over theory, it is neither a necessary nor a sufficient condition to explain all of the cytogenetic data.

The breakage-and-repair hypothesis is generally supported by the observation of elevated chromosome damage, and the ubiquitousness of the mechanism [37]. But failure to find any significant frequencies of aberrations other than chromatid breaks and exchanges, plus multicentric chromosomes, implies sharp restrictions both on how chromosomes might be broken and how they might be repaired. Such a restriction placed on a physical agent like radiation or radiomimetic chemicals is difficult to imagine. The inverted ratio of breaks to exchanges seems to require that the repair process be much more active than in normal cells. No

radiothymidine labelling could be found during the normal time of the G_2 phase in cultured FA cells. This is further evidence that "unscheduled" DNA synthesis is not involved in chromosome repair. The average time required to repair radiation-induced chromosome breaks in cultured FA lymphocytes during G_1 was found to be normal (Figure 24). Some very rapid repair was implied as well. Rapid repair should heal simple breaks fast enough to reduce the frequency of exchanges; slow repair would reduce slightly the observable breaks without a large increase in exchanges. Therefore the repair time data do not assist the breakage-and-repair hypothesis to explain the inverted break/exchange ratio.

An extended duration of the G_2 phase of the FA cell cycle was implied by failure of a large fraction of dividing cells to label with radiothymidine (Figure 15), and by the assumption that <u>labelled</u> cells consisted of two populations (Figure 19). However the near normalcy of the FA thymidine labelling fraction (Figures 13 and 15, and Table 6) suggested that FA cells had a near-normal S/T_G ratio. A normal T_G (assumed in Table 6) then would require the cells to have a normal S. But if G_2 were lengthened, then S might have to be shortened; this would not permit a normal S/T_G ratio. A similar line of reasoning would require a

shortened G_1 if S were normal; this possibility is not excluded by the data. It is very much simpler to assume that the unlabelled cells were those with a long G_2; this also would not allow a normal labelling fraction. Altogether too many assumptions must be made to obtain an increase of the time available to FA cells in which to repair chromosome damage, and it is safest to conclude that increased time available for repair was not shown.

A variant of the abnormal repair requirement was the hypothesis of hexokinase deficiency in FA cells. This was easily disposed of, as the FA cells had at least normal hexokinase activity (Table 5).

By themselves the numerous chromatid breaks support the hypothesis of increased breakage. But increased breakage requires either or both of an increase in the breaking agent or an increase in susceptibility to breakage. The former is simply not in evidence. Exposure to such an agent must have been greatly reduced in washed cultured cells; yet the aberration frequency after several divisions was nearly the same as at the outset (Figure 25). The remaining possibility, increased susceptibility to damage, was demonstrated plausibly in cultured lymphocytes of other FA cases [54]. Susceptibility to observable radiation-induced chromosome damage may be conditioned by a host of variables. The hexokinase deficiency in

some but not all FA cases [112,113] comes to mind, as well as conditions of culture and the presence of plasma [118,134]. In any event, the susceptibility of the present FA case's chromosomes to radiation-induced damage during G_1 and G_2 could not be demonstrated (Tables 10 and 11).

Summarizing the foregoing, it is apparent that the breakage-and-repair theory explains some of the experimental observations, but does not easily explain them all. Corollary requirements of the theory are not demonstrated. As with the somatic crossing-over hypothesis, breakage-and-reunion is not a sufficient condition to explain all the data. That it may be a necessary condition for some experimental results continues plausible, and also unproven.

The exchange hypothesis remains. It does not require an increased susceptibility to chromosome breakage, nor does it require increased time for or efficiency of repair. The inverted break/exchange ratio found in early examinations is explained, as "breaks" are derivatives of incomplete exchanges rather than prerequisite for aberrations. But the theory contains no elements which would permit restriction of aberrations to the few forms observed. Thus this hypothesis also is insufficient to explain all of the data.

The breakage-and-repair and the exchange hypotheses were derived originally from experiments with

physical agents such as radiation and radiomimetic chemicals. Some of the difficulties in applying these theories to the present data arise from the assumption that some such randomly-acting agent is involved in the etiology of FA. The consequences of assuming action by a non-randomly acting substance may be worth exploring.

Many non-radiomimetic chemicals cause preferentially chromatid-type damage and elevated chromatid exchange frequencies like those found in FA cases [e.g. 85]. If exposure to an exogenous chemical in vivo were responsible for the FA chromosome aberrations, it is difficult to see how its action could be restricted to lymphocytes, with no aberration induction in marrow cells (Table 12). Further, second and later divisions in vitro might be expected to show lower aberration frequencies than the first; this is denied by Figure 25. It does not seem likely that an exogenous chemical could produce the results described.

Bacterial involvement may be ruled out; there is not the slightest shred of evidence for bacterial activity in any aspect of the etiology of FA from the grossest physical changes down to the cellular level. On the same grounds, bacterial products cannot be a factor.

Some product of the FA cells themselves was postulated to explain the chromatid breaks found in cross

incubation experiments (this report). But such a product should accumulate in the closed tissue culture system and cause a rise in aberration frequency with increased time in culture. Figure 25 is a denial of this proposition, and a cell product is unlikely to be the significant factor in FA chromosome abnormalities.

Viral activity remains to be considered. The action of a virus has been suggested already to explain the late-occurring anemia in FA [128] and the frequently occurring leukemia [116]. Severe viral infections often have preceded the anemic phase [128], and this was true in at least three of the four FA cases described in this report. Viruses also can produce a pattern of chromosome aberrations like those reported here. Sotomayor observed elevated chromatid exchanges along with chromatid breaks in swine which had been innoculated prophylacticly with a virus [124]. Gripenburg found that cultured leukocytes of patients with viral infections often displayed greatly increased frequencies of chromatid exchanges, with smaller increases in other aberrations [46]. She did not note the significance of the inverted break/exchange ratio, although she specifically noted its occurrence.

The action of a virus could explain both the poor survival of FA lymphocytes *in vitro* and its delayed onset (Figure 11). Bouroncle *et al.* found maximum viral infectivity at 48 hours in PHA-stimulated cultures of

human lymphocytes [12], and this coincides with the onset of decreased survival seen in Figure 11. Gripenburg referred to a chromosome breaking substance in the plasma of patients with viral conditions [46]. If cultured FA lymphocytes are viewed as "patients with viral conditions" then the cross-incubation results are explicable (Table 9). The decline in chromatid exchange frequency throughout the course of the propositus's disease (Figure 27) is consistent with the notion of therapy-induced cessation of this particular viral manifestation.

Figure 26 shows simple chromatid breaks do not decline as do the chromatid exchanges; Figure 27 shows the exchange decline is consistent with the disappearance of a lymphocyte population. These observations suggest the existence of two populations of lymphocytes: one very susceptible to (postulated) viral activity and therapy effects, the other less easily affected by either viruses or therapy. This is not a strong argument, but two lines of evidence support it. The early mitotic peaks in FA lymphocyte cultures are very labile (Figures 9 and 10), and this may represent some insult to Bender's "sensitive" population of lymphocytes [7]. The argument for an abnormal lymphocyte population with respect to either or both of cell cycle parameters and radiothymidine labelling has been given earlier; both effects could be produced by viral

infection of cells. Restriction of marked viral activity to a single subpopulation is possible; medical and veterinary literature abounds with accounts of viral pathology associated with or restricted to a single organ or tissue type. In a purely speculative way, it may be worth considering the "leukovirus" found only in cultured lymphocytes from about one-third of normal humans [74,120]. This herpes-like virus has no known pathological effect, but there is no reason to believe it would not acquire one given the appropriate cellular genetic background. A search for virus by direct electron microscopy of fresh and cultured FA lymphocytes gave no evidence for the presence of virus (Figures 21 and 22). Unfortunately this sort of negative result is not conclusive one way or the other.

The breakage-and-repair hypothesis as applied to FA chromosome abnormalities is not materially enhanced by invoking viral action rather than a physical agency to initiate chromosome damage. All the arguments given previously would still apply. However the exchange hypothesis gains added credence: chromatid exchanges <u>are</u> more frequent in the lymphocytes of viral infectees [46]. Intracellular viral effects are not at all random, and the restriction of chromosome aberrations to certain classes is imaginable. If this hypothesis is true, specificity of viral action in producing certain classes of chromosome damage would be a good sub-

ject for further research.

It must be emphasized that the action of a virus, or any other exogenous agent, has not been proven in FA either in this report or anywhere else. Viral action is merely consistent with the experimental and clinical evidence. But if a virus is involved in the etiology of FA, what of the recessive gene hypothesis which is so widely held?

The pertinent data in this report are consistent with the recessive gene hypothesis, with three exceptions. The heavily labelled cells (Figure 20) seen in both the FA case and her mother are not evident in the father's cells. A severe disturbance in the FA cases's cell cycle was noted (Figures 12 and 14), and the mother's G_1 phase was probably lengthened (Table 7) among other possibilities (Figure 15); no similar changes could be found in the father's cells. The father's kindred showed a very much greater rate of infant mortality than the mother's (Table 1). If these phenomena are related to the entire FA syndrome, they are not what would be expected if FA were due to a single fully recessive gene. Sex-linkage or sex-limitation of features of FA could explain the three differences cited above, but there is no evidence of linkage or limitation in any aspect of FA.

On the other hand, the deficit in radiothymidine labelling seen among FA cells was also seen in a small

way among the cells of both parents. This would be expected on the recessive gene hypothesis, and indeed the phenomenon was suggested as the basis for a simple test to detect the FA heterozygote.

The recessive gene theory is not always supported strongly by the reports of others; in some cases the theory is greatly weakened. At least two cases of FA children born to FA mothers and normal fathers have been described [60,87]. If homozygosity for a recessive gene is responsible for FA, then both normal fathers would have had to be heterozygotes. The great rarity of FA suggests a gene frequency which is very low, and thus heterozygotes would be fairly uncommon. Matings of homozygotes (FA mothers) to heterozygotes ("normal" fathers) should be rare. Yet of the 100 or fewer matings from which published FA cases have been reported [44,138,140,141], two were of this sort.

Reinhold et al. provided the first formal statistical test of the recessive gene hypothesis; they concluded their pedigree data from 14 families were consistent with the hypothesis [98]. But Weicker found that recessive inheritance could explain only the segregation ratio obtained by counting sibships in order of birth rank up to and including the first FA sib [138]. Additional sibs after the first FA case were more likely to be FA cases themselves than could be accomodated under the hypothesis of recessive inheri-

tance; the "runs" observed were highly improbable (P=0.004). The same phenomenon was found later by Gmyrek and Syllm-Rapoport in their review of 129 FA cases [44]. In both instances, the authors believed strict recessive inheritance could not be supported by the data. The "runs" were explained by Gmyrek and Syllm-Rapoport as "...due to peristatic factors produced by the first diseased child...", while Weicker urged attention be paid to the similarity with mother-child blood group incompatibility. Both effects of course would be non-genetic influences operating in FA. In passing, it should be noted that all the multiple-case sibships shown by Reinhold et al. demonstrate the "runs" of FA cases [79], as does the family which is the subject of this report (Figure 3).

Besides Weicker, and Gmyrek and Syllm-Rapoport, several authors have expressed doubts about the recessive nature of FA [27,35,41,116]. In light of all the foregoing it would be highly desirable to re-examine the segregation ratio using a larger series of families. Unfortunately few authors have provided adequate pedigrees in their case reports. Li and Mantel provided a new method of estimating the segregation ratio under complete ascertainment [64]. With this assumption, applying their "cast out the singles" method to the pedigree data of Reinhold et al. shows the data remain consistent with recessive inheritance (chi-squared=

1.66; $P \cong 0.2$). But the segregation ratio becomes 1 in 3 rather than the 1 in 5 cited by Reinhold et al. Clearly such a ratio also will be compatible with other modes of inheritance: for example, inheritance of a dominant gene with reduced penetrance, with varying effects in the heterozygote and full lethality in the homozygous dominant individual. The theory of recessive inheritance is sufficient, but not necessary to explain the incidence of FA.

The proposition of full lethality associated with some combination of FA genes receives a degree of support from the high incidence of infant mortality noted by Nilsson [79] and also seen in the kindred reported here (Table 1). Data on miscarriages and abortions in FA families would be most welcome, but they are seldom reported and have proven difficult to obtain in the present study.

Many of the features of FA are seen also in congenital telangiectatic erythema (Bloom's Syndrome). This disease is characterized, among other things, by stunted growth with some malformations, increased risk of leukemia, and elevated frequencies of chromatid exchanges in cultured lymphocytes [42,105]. It is virtually certain to be caused by homozygosity for a simple recessive gene [40]. Anemia is not part of the syndrome, and German et al have suggested homology of this condition with that of the non-anemic but mal-

formed individuals found in FA kindreds [42]. The ratio of chromatid exchanges to simple chromatid breaks is inverted from expectation, just as in FA [42,105]. It would be most interesting to repeat the experiments reported here, using lymphocytes from cases of Bloom's Syndrome. If any direct relationship of Bloom's Syndrome to FA should be demonstrated, certainly the prevailing view of the recessive nature of FA would require modification.

Fanconi's Anemia clearly is familial, and in all likelihood it has a strong true genetic component in its causation. But equally clearly FA is not a simple genetic disease; it has complex facets which have been examined only imperfectly. If anything, the present series of investigations has revealed only more complexity than was suspected before.

The present experimental studies are preliminary in nature. They concern a single case of FA, and the results must be confirmed or denied by studies of additional cases. But this work has produced and supported a few hypotheses which can be the framework around which future studies can be designed. Additional cases of FA are being sought actively, and the present case continues to be studied at intervals.

SUMMARY

The genetic and cytogenetic literature concerning Fanconi's Anemia (FA) is reviewed. A case is made against the prevailing simplistic view of the etiology of FA. A rationale for further study is given.

An unreported case of FA is presented. Physical and clinical descriptions are given for the FA case, her normal sib, both normal parents, and three deceased FA sibs.

A pedigree of the kindred is given, containing 188 individuals in 7 generations. There is no parental consanguinity. The incidence of neoplasia and diabetes in the kindred is not elevated, but ascertainment is incomplete. Diabetes is present in the proposition's immediate antecedents. Infant mortality is elevated, and is 21% of live births in the paternal kindred.

Marrow cells of the propositus contained no chromosome abnormalities during the 5 1/2 year period of observation. A small proportion of marrow cells contained numerous micronuclei.

Cultured FA lymphocytes contained increased chromatid exchanges, gaps, and breaks, with multicentric chromosomes; there were virtually no other abnormalities. Chromatid exchanges were more common initially than chromatid breaks; this ratio reversed by the end of the study period. The frequency of chromatid ex-

changes declined exponentially through the course of treatment, with a half-time similar to the half-life of lymphocytes. Multicentric chromosomes were always less frequent than exchanges; they also declined with time. Chromatid breaks did not decline during the course of the disease. Endoreduplication was not common throughout the 5 1/2 year study, but was elevated following termination of treatment.

The frequency of chromosome aberrations did not decline in the cultured FA lymphocytes with time in culture. This was interpreted as de novo creation of abnormalities in each cell generation.

Homologous chromosomes were involved frequently in chromatid interchanges, but non-homologous exchange association was common. Long chromosomes were more common than short in chromatid exchanges. The increase above expectation may follow a power relationship.

Mitotic index determinations on cultured FA lymphocytes revealed inconsistent and abnormal patterns, suggesting multiple cell populations or cell cycle abnormalities.

In vitro survival of FA lymphocytes is reduced in two phases, one prominent after 48 hours of culture. Some of the reduced survival shown by dye-exclusion is spurious, suggesting a defect in cell membrane integrity.

Lymphocytes from FA and normal individuals were cultured in the presence of each other's plasma; no changes in frequencies of chromosome abnormalities were observed. The experiment repeated with "conditioned" medium produced a seven-fold increase of simple chromatid breaks in normal cells.

Electron micrographs of fresh FA lymphocytes were not different from those of normal individuals. Micrographs of cultured FA lymphocytes showed unusual lacunae in the endoplasmic reticulum and an accumulation of granules believed to be glycogen. Mitochondria were abnormally large, with reduced and unusually arranged cristae; ribosomes were reduced.

Cell cycle analysis was performed on lymphocytes from all family members. The mother's G_1 phase probably was prolonged; the durations of either or both of S and G_1 were variable in both parents' cells. The normal sib had normal cell cycle parameters. The FA case's cell cycle times could not be determined. A large fraction of her dividing cells failed to label with radiothymidine; a much smaller fraction of both parents' cells also failed to label. The mother's cells showed some labelling late in G_2. Both the FA case and her mother, but not her father or normal sib, had a proportion of cells which labelled intensely. Additional evidence for a membrane defect in FA cells was obtained. Two populations of labelling FA cells

were postulated; one would have an abnormal cell cycle.

Failure of cultured lymphocytes to label with radiothymidine was offered as a clinically useful test for detection of the FA carrier.

Both hexokinase and thymidine kinase activities were normal or elevated in cells of the FA case and her immediate family.

The G_1 chromosome repair time of the FA case was normal on the average, but the experimental results suggested either multiple cell populations or great variability of the repair time. Repair times determined for the other family members were the same as the controls'.

The rate of chromosome-type aberration induction by x-rays during the first G_1 in cultured FA lymphocytes was not elevated above control values. The rate of chromatid-type aberration-induction in the last G_2 in FA lymphocytes was not elevated above control values. Determinations performed on family members' cells showed no difference from control values.

On two occasions, almost three years apart, no significant aberrations were found in the chromosomes of cultured lymphocytes from the FA case's parents and normal sib.

The cytogenetic abnormalities in cultured FA lymphocytes occur in a relatively simple pattern which

is not consistent with the hypothesis of chromosome fragility and subsequent mis-repair. The "exchange" hypothesis of chromosome aberrations is more in accord with the bulk of the observational data given in this report.

It is shown that the action on FA chromosomes of a physical agent is inconsistent with both observational and experimental evidence. The action of a virus, especially as the initiating agent in the exchange hypothesis, is consistent with but not proven by the data provided.

The recessive gene hypothesis of FA causation was questioned. A more complex genetic basis for FA was considered likely.

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Lastly, and most certainly not the least, I thank the patient and her family. Grounding their actions in the belief that scientific study of their family might produce useful insight into the cause of FA, all have been most cooperative. On 21 separate occasions the family has come to the laboratory to yield up samples of blood or marrow; often at times to suit the convenience of the experiment, occasionally to have the experiment fail. Without their earnest cooperation and understanding this work could not have been performed. To them most of all go both my gratitude and my sympathy.

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APPENDIX I. Observations listed by time since diagnosis of FA in the propositus.

Date	Years since diagnosis	Studies performed[1]
25 Oct.1967	0.0	Marrow karyotype.
13 Nov. "	0.1	Lymphocyte karyotype.
24 Jun.1968	0.8	Lymphocyte karyotype (family).
27 Sept."	0.9	Marrow karyotype; Hexokinase (family).
5 Mar.1969	1.4	Mitotic index; Lymphocyte karyotype.
9 Jun. "	1.6	Mitotic index.
22 Jul. "	1.7	G_1 chromosome repair time; Lymphocyte and marrow karyotype.
4 Aug. "	1.8	Mitotic index; G_2 chromosome breakage; Lymphocyte karyotype.
2 Sept. "	1.9	Mitotic index.
24 Sept."	1.9	Mitotic index.
20 Oct. "	2.0	Cell viability; Cell cycle analysis.
11 Feb.1970	2.3	Cell viability; Cross-incubation; Electron microscopy; Lymphocyte karyotype.
11 Mar. "	2.4	Aberrations vs. time <u>in vitro</u>; Cell viability; Hexokinase (family); Cell cycle analysis (family); G_1 chromosome repair time; (PH, EH, KH).
12 May "	2.6	G_1 chromosome repair time (PH, HH).
26 Feb.1971	3.3	Thymidine kinase (family).
10 Mar. "	3.4	Lymphocyte karyotype (family).
10 Oct.1972	5.0	Lymphocyte karyotype.
11 Oct. "	5.0	Marrow karyotype.
24 Jan.1973	5.3	Lymphocyte karyotype.

[1]Studies were on the propositus only unless noted. Family = both parents, the normal sib, and propositus; PH = the propositus; HH = father; EH = mother; KH = normal sib.

APPENDIX II. Genetic Markers: All Family Members

Determination		Date	PH	EH	HH	KH
Serum Lactic Acid Dehydrogenase (LDH) Isoenzymes (% of total)[1]		11-29-67				
	Fraction V		3.4	7.4	5.5	5.8
	IV		6.2	13.0	14.0	20.3
	III		15.2	20.7	16.5	14.8
	II		42.0	38.6	42.0	37.0
	I		33.2	20.3	22.0	22.1
Haptoglobin Types[2]		3-1-68	2-1	2-2	2-1	2-2
Blood Groups[3]		12-5-67				
Factors	A		+	+	+	+
	B		-	-	-	-
	C		-	-	-	-
	D		-	-	-	-
	D^u		-	-	-	-
	E		+	-	+	+
	c		+	+	+	+
	e		+	+	+	+
	MN		+	+	+	+
	S		+	+	-	-
	P		-	-	-	-
	Fy^a		+	+	-	+
	Fy^b		+	+	+	+
	K		-	-	-	-
	Le^a		-	-	-	-
	Le^b		+	+	+	+

APPENDIX II. (Continued)

Determination		Date	PH	EH	HH	KH
Blood Groups (Continued)		12-5-67				
	ABO		AA(or) AO	AA(or) AO	AA(or) AO	AA(or) AO
			cdE	cde	cdE	cdE
	Rh		cde	cde	cde	cde
			D^u-	D^u-	D^u-	D^u-
	MNS		MNSs	MNSs	MNss	MNss
	KELL		kk	kk	kk	kk
	P		pp	pp	pp	pp
	DUFFY		Fy^aFy^b	Fy^aFy^b	Fy^bFy^b	Fy^aFy^b
	LEWIS		Le^bLe^b	Le^bLe^b	Le^bLe^b	Le^bLe^b

[1]San Francisco General Hospital, San Francisco, California.
[2]Children's Hospital, San Francisco, California.
[3]Alameda-Contra Costa Medical Association Blood Bank, Oakland, California

PH = Propositus; EH = mother; HH = father; KH = normal sib.

APPENDIX III. Clinical Laboratory Determinations[1]

Determination	Date	Lab. Normal	PH	EH	HH	KH
Glucose-6-Phosphate Dehydrogenase (G6PD) Activity (minutes to completion)	1-24-68		60[2]			
	11-29-67			85	85	85
Fetal Hemoglobin (% of total)	11-3-67	0.5-1.7	12.8			
	11-29-67			1.3	1.1	1.4
Total Serum Protein (gm%)	11-29-67	5.9-7.5	6.1	6.3	6.6	6.8
Serum Protein Electrophoresis (% of total)	11-29-67					
Albumin			63.6	54.0	60.4	53.6
α_1			3.0	3.5	2.3	3.1
α_2			7.1	16.1	10.5	13.4
$\beta_1 + \beta_2$			18.2	17.2	16.3	16.5
γ			8.1	9.2	10.5	13.4

[1]All clinical laboratory determinations were performed at the Donner Laboratory, except Coombs and VDRL, which were performed at the California State Department of Public Health, Berkeley, California.

[2]RBC concentration increased to give normal hematocrit.

APPENDIX III. (Continued)

Determination	Date	Lab. Normal	PH	EH	HH	KH
Mean Corpuscular Hemoglobin (MCH: µµg)	various[1]	27-32	28-42	32	33	32
" " " Concentration (MCHC: %)	"	33-38	31-41	30	31	29
" " Volume (MCV: μ^3)	"	80-94	72-115	95	95	91
Reticulocytes (% of RBC)	"	0.2-1.5	0.2-6.4	1.0	0.8	1.6
Hemoglobin (gm%)	"		Fig. 1	13.0	16.3	13.6
Red Blood Cells (RBC x $10^6/mm^3$)	"		"	4.31	5.22	4.65
White Blood Cells (WBC/mm^3)	"		"	7800	6800	6300
Neutrophils (%)				65	65	57
Lymphocytes (%)	"		"	26	22	37
Others (%)				9	13	6
Platelets (x 1000/mm^3)	"	150-500	"	435	310	335
Serum Iron (SI: µg%)	10-25-67	70-170	197			
	9-9-70		164			
Latent Iron-binding Capacity (LIBC: µg%)	10-25-67	230	228			
	9-9-70		307			
Total Iron-binding Capacity (TIBC: µg%)	10-25-67	300-400	425			
	9-9-70		471			
Iron Turnover in Plasma (mg/kg/day)	10-30-67		0.634			
Protein-Bound Iodine (PBI: µg%)	10-30-67	3.8-7.8	21.3			
Total Protein-Bound Iodine (TPBI: µg%)	10-30-67		39.6			

[1]11-29-67 for HH, EH, and KH; various dates for PH.

APPENDIX III. (Continued)

Determination	Date	Lab. Normal	PH	EH	HH	KH
Blood Urea Nitrogen (BUN:mg%)	10-30-67	10-18	14.0			
Serum Uric Acid (mg%)	10-30-67	2-6	2.2			
Plasma Erythropoietin (IRP units/ml)	11-8-67	0.003	3.6			
Leukocyte Alkaline Phosphatase (LAP:score)	10-30-67	24-48	246			
Direct Coombs	10-30-67	---	Neg.			
VDRL	10-26-67	---	Neg.			
Prothrombin Time(sec.)	9-25-68	12.5	14.5			
Coagulation Time (Lee-White)(min.)	9-18-68	3-10	12			
Glucose Tolerance	1-2-68	---	Normal			
L-E Prep.	10-30-67	---	Neg.			
Hemoglobin Electrophoresis	10-30-67	---	A + F			

PH = Propositus; EH = Mother; HH = Father; KH = Normal sib.

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