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Chromosome Abnormalities in Mice With Hertwig's Anemia

By Janan T. Eppig and Jane E. Barker

Mice with the recessive hereditary disease, Hertwig's anemia (*an/an*), exhibit a persistent mild macrocytic anemia and reduced fertility. We examined mitotic figures from bone marrow and kidney cells of adult mice and from liver cells of fetal mice that were genetically normal or had Hertwig's anemia. Uniformly normal mitotic figures were observed in the nonanemic mice (+/+ or +/*an*). In contrast, 5% to 15% of the mitotic figures were abnormal in mice homozyous for Hertwig's anemia (*an/an*). These aberrant cells were hyperploid, containing more than the normal complement of c40 chromosomes, but fewer than 80 chromosomes. Cells with abnormal numbers of chromosomes may show decreased viability or proliferative capacity. The occurrence of such abnormal cells in *an/an* mice could explain (1) the loss of progenitor stem cells during erythroid maturation, resulting in an anemic phenotype; and (2) the depletion of germ cells during ontogeny, resulting in reduced fertility.

HERTWIG'S ANEMIA (gene symbol *an*) is a recessive mutation on chromosome 4 that arose in 1939 in offspring of irradiated mice.[1] Homozygous *an/an* mice have a mild macrocytic anemia and reduced fertility. White and red blood cell values are lower than normal throughout life. Young *an/an* females are able to conceive but have fewer oocytes than +/+ females and are unable to deliver their young. Males are sterile. Aging *an/an* mice have a high incidence of histiocytic sarcoma (reviewed).[2,3]

The defect in *an/an* cells is less severe than in the other known murine macrocytic anemias, *W* (dominant white spotting) and *Sl* (steel). W/W^v animals have an intrinsic defect in their hemopoietic stem cells and can be cured of anemia by supplanting their hemopoietic system with injected +/+ bone marrow cells.[4,5] Sl/Sl^d animals exhibit a microenvironmental defect in that they cannot be cured by +/+ bone marrow cells,[6] but Sl/Sl^d bone marrow can be used to cure W/W^v anemics.[7] Barker et al[8] showed that +/+ bone marrow cells injected into mice with Hertwig's anemia did not cure the anemia unless the *an/an* mouse was first lethally irradiated to destroy the native stem cells. Moreover, *an/an* cells co-injected with +/+ cells into lethally irradiated mice are able, at reduced efficiency, to compete in repopulating the host's hemopoietic system. The slight disadvantage *an/an* marrow cells have in competing with +/+ cells was postulated as a decreased relative number of stem cells among the *an/an* marrow cells or a lower rate of proliferation of the *an/an* stem cells.

An examination of the number of cells in various precursor hemopoietic stem cell populations of *an/an* versus +/+ animals demonstrated that as the stem cells mature, the difference between *an/an* and +/+ cells becomes greater.[9] At all stages examined, the *an/an* stem cell population is less than the +/+ stem cell population. However, when comparing +/+ and *an/an* stem cell populations, the relative difference between the number of CFU-S (colony forming units—spleen) is less than the difference between the number of BFU-E (burst forming units—erythroid); and the difference between the number of BFU-E is less than the difference between the number of CFU-E (colony forming units—erythroid). This indicates a progressive loss of *an/an* precursor stem cells during hemopoietic maturation.

In the present study, mitotic figures from various tissues of *an/an*, +/*an*, and +/+ mice were examined. The rationale for this approach was as follows: (1) Hertwig's anemia arose in offspring of irradiated mice. Because radiation is known to be both a mutagen and a chromosome breaking agent, the *an* mutation could be a small chromosomal deletion; and (2) abnormal mitotic figures and many degenerating cells were observed in gonads of presumed *an/an* fetuses (12 to 13 days gestation).[3]

Results presented here show that a proportion, generally 5% to 15%, of the mitotic figures from various tissues of *an/an* mice have a hyperploid chromosome number. No consistent pattern of extra chromosomes was observed. Cells with an abnormal number of chromosomes may have reduced viability or proliferative rate. In addition, in some metaphases from bone marrow cells cultured in vitro for one week, the centromeres of two or more chromosomes appeared attached to or associated with one another in pairs or multiples. This observed chromosome association could be a prelude to improper division and result in cells with abnormal numbers of chromosomes. We hypothesize that the *an/an* mice may have an initially normal number of pluripotential hemopoietic stem cells that divide at a normal rate to produce precursor

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hemopoietic cells. This precursor cell population becomes increasingly small as chromosomally abnormal cells are produced and die during maturation. Such a process could produce the mild anemic phenotype of the *an/an* mice.

MATERIALS AND METHODS

Mice

Crosses were made between mice of the WB/Re and C57BL/6J strains, heterozygous for the genes *an* and B^{lt} (light), a linked codominant coat color marker. Hertwig's anemic mice produced from this cross were $WBB6F_1-B^{lt}$ *an*/B^{lt}*an*. Most $WBB6F_1$ non-anemic mice were of genotypes ++/B^{lt}*an* or ++/++. Because crossing over occurs between the genes B^{lt} and *an* with a frequency of approximately 0.10,[1] blood values of all adult light-colored animals were tested to ensure that they were *an/an*. Red blood cell counts, hematocrits, and mean cell volumes were compared with published values for normal and *an/an* animals.[2,3] Fetuses (14 to 15 days old) were from a cross of $WBB6F_1-B^{lt}$*an*/B^{lt}*an* females and $WBB6F_1$ − ++/B^{lt}*an* males. Fetal genotype was determined by the pale appearance and low RBC counts of *an/an* fetuses when compared with +/*an* fetuses.

Cell Cultures

Bone marrow cells were cultured as described by Dexter et al.[10] In brief, bone marrow cells were flushed from the femur into medium (Fischer's leukemic mix, GIBCO 320-1475, supplemented with 20% horse serum and 2 mL/100 mL penicillin-streptomycin, GIBCO 600-5140, GIBCO, Grand Island, NY). Cells were dispersed with the aid of a syringe and 20-gauge needle. Culture flasks (25 mL) were seeded with 10^7 cells in 10 mL medium, gassed thoroughly with 5% CO_2 plus air, and incubated at 33 °C for five to seven days.

Kidney cells were cultured as follows. Kidneys were dissected from adult mice and the kidney capsule carefully removed. Each kidney was cut into small pieces of approximately 1 μL and the pieces placed in a trypsinizing flask with 5 to 10 mL of 1.0% trypsin (GIBCO 610-5090), 0.1% collagenase (type II, Sigma C-6885, Sigma Chemical Co, St Louis) in 1× sterile phosphate buffered saline (PBS, GIBCO 310-4190). The flasks were placed in a shaking water bath (37 °C) for 30 minutes. The contents of the flasks were transferred to sterile conical centrifuge tubes and allowed to settle for two to three minutes. The supernatant was removed with a Pasteur pipette and 4 to 5 mL of sterile PBS added vigorously to agitate the settled chunks of tissue. This washing procedure was repeated twice. A final wash was done using culture medium (Eagle's minimal essential medium with Earle's salts, plus 10% fetal calf serum and 0.5 mL penicillin-streptomycin). Each culture dish (60 mm) was seeded with the tissue from one half of a kidney in 2 mL medium and incubated at 37 °C in humidified 5% CO_2 plus air. The cultures were derived from outgrowths of the tubule tissue fragments. At 48 hours, the contents of each culture dish were transferred to a second culture dish to allow the remaining loose tubule fragments to settle and grow. The medium in the first culture dishes was replaced with fresh medium. Culture medium was replaced every 48 hours. The kidney cultures were harvested at subconfluency, after four to six days of culture, as described below.

Mitotic Chromosomes

Mitotic chromosomes from fetal liver were prepared as described by Eicher and Washburn.[11] Livers were excised from 14- to 15-day fetuses and placed individually in 15-mL plastic conical centrifuge tubes containing 2 mL warm (37 °C) EDTA-colchicine buffer (8 g NaCl, 0.2 g KH_2PO_4, 0.2 g KCl, 1.15 g Na_2HPO_4, 0.2 g EDTA disodium salt per liter H_2O plus 0.05% colchicine). After the cells were dispersed with a syringe and 20-gauge needle, 8 to 10 mL additional EDTA-colchicine buffer was added and the cells incubated (37 °C) for ten to 15 minutes. The cells were centrifuged (200 g) and the supernatant removed. Cells were resuspended in warm (37 °C) 0.75 mol/L KCl and incubated at 37 °C for 20 to 30 minutes. The cells were centrifuged, the supernatant removed, and fixative (methanol:glacial acetic acid, 3:1) added. Following two washes with fixative, the cells were resuspended in fixative to a final volume of 2 mL and dropped onto clean wet slides.

Mitotic chromosomes from fresh bone marrow were prepared in an identical manner using marrow flushed from the femur.

Bone marrow cultures were treated with 0.02 mL and kidney cultures with 0.1 mL of colchicine (50 μg/mL 0.85% NaCl) per milliliter of culture medium plus cells for one to two hours before harvest. Kidney cells were dislodged from the culture dish by a 30-minute treatment with the EDTA-colchicine buffer of Eicher and Washburn.[11] The culture dishes were also physically scraped with the wide end of a Pasteur pipette to help dislodge the cells. Bone marrow and kidney culture cells were harvested following the method used for fetal liver with the exception that cultured kidney cells received only a 10-minute treatment with 0.075 mol/L KCl.

Trypsin-Giemsa Banding of Chromosomes

Trypsin-Giemsa banding of chromosomes was accomplished by the method of Eicher and Washburn.[11] Three coplin jars were assembled containing (1) 50 mL physiologic saline plus 4 mL trypsin 2.5% (10×, GIBCO 610-5090); (2) physiologic saline; and (3) 50 mL 0.05 mol/L KH_2PO_4 (pH 7.0 with NaOH) plus 2 mL Giemsa stain (Gurr R66). Slides were dipped into the first solution and gently moved to and fro for eight to ten seconds. Slides were transferred to the physiologic saline and washed thoroughly, then transferred to Giemsa solution for ten minutes, rinsed with water, and air dried.

RESULTS

Table 1 shows the distribution of chromosome numbers in metaphase preparations from fresh bone marrow of adult mice ranging in age from two to 18 months. In evaluating metaphases, obviously polyploid cells and incomplete mitotic figures were disregarded. It is possible that some mitotic figures classified as incomplete (fewer than 40 chromosomes) were the result of abnormal cell divisions, but such cells were not counted in this study because they could be the result of technical artifact. Polyploid cells are found among normal bone marrow cells and, therefore, were assumed to be normal. Since both incomplete and polyploid cells from *an/an* individuals could be the result of abnormal cell division, this method of counting should underestimate the *an/an* cell abnormality. Animals of +/+ or +/*an* genotype were chromosomally normal. A variable number of mitotic figures from mice with Hertwig's anemia (*an/an*) contained abnormal numbers of chromosomes. A

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Table 1. Chromosome Number in Metaphases From Fresh Bone Marrow

Mouse			Number of Chromosomes*					Percentage of Abnormal Metaphases
Sex	Age (mo)	Genotype	39	40	41-50	51-60	61-79	
M	2	+/*an*		24				0
F	8	+/*an*		21				0
M	14	W/W^v		17				0
M	15	+/+	1	28				0
M	18	+/+	1	32				0
M	18	+/+		50				0
M	2	*an/an*		25	5	3	2	28.6
F	4	*an/an*		28	1	3		12.5
F	6	*an/an*	1	43		4	2	12.0
M	6	*an/an*	8	51	3	2	3	11.9
M	6	*an/an*		69		3	2	6.8
M	6	*an/an*		46	2	3	3	14.8
F	8	*an/an*		29	1	4	2	19.4
M	14	*an/an*	4	46	2	2	3	12.3
M	15	*an/an*		45			1	2.2
M	15	*an/an*		28	2			6.7
M	17	*an/an*		35	1	2	1	10.3
M	17	*an/an*		35		2	1	7.9
M	17	*an/an*		23		1	2	11.5
M	17	*an/an*		47		8	2	17.5
M	17	*an/an*		33	2	1	1	10.8
M	17	*an/an*		31	2			6.1

*Metaphases with more than 80 chromosomes or less than 39 chromosomes were disregarded. Nonpolyploid cells with more than 40 chromosomes were counted as abnormal. Cells with 39 chromosomes were considered "normal" with artifactual chromosome loss. By disregarding metaphase spreads with small numbers of chromosomes and counting 39 chromosomes as "normal," the percentage of abnormal metaphases is underestimated.

karyotype of an abnormal mitotic figure from *an/an* bone marrow is shown in Fig 1.

Cultured cells from adult mice showed a similar pattern of chromosome abnormalities. Table 2 shows the distribution of chromosome numbers in mitotic figures from bone marrow cell cultures and kidney cultures of four *an/an* and two +/+ mice. Figure 2 shows a karyotype of an abnormal mitotic figure from cultured *an/an* kidney cells.

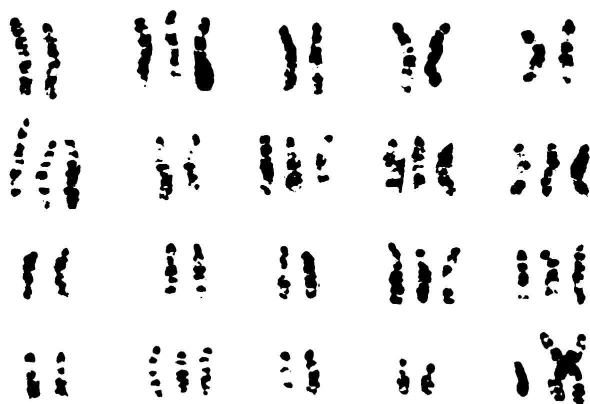

Fig 1. Karyotype of abnormal mitotic figure (49 chromosomes) from fresh *an/an* bone marrow cell. There is one extra copy of chromosomes 6, 8, 9, 10, 14, 15, and 17. In addition, there are two extra copies of chromosome 2 (one lies over the *X*). One of the two chromosome 6 has extra G-banded material on the proximal end.

Some chromosomes in metaphases from cultured bone marrow cells of *an/an* mice appeared to be attached or associated at their centromeric ends, although the number of chromosome arms present was normal. Chromosomes appeared to be associated at their centromeric ends in pairs or multiples (Fig 3). Cells from +/+ mice occasionally appeared to have one or two associated chromosomes but at a very low frequency and never in multiples. This chromosome association could be a prelude to abnormal cell division.

Table 3 shows the distribution of chromosome numbers in mitotic figures from livers of 14- to 15-day fetuses. Again, mitotic figures from all normal fetuses (+/*an*) contained a normal chromosome number, and a proportion of the mitotic figures from the *an/an* fetuses had more than 40 chromosomes. Among the *an/an* fetuses, the percentage of cells with abnormal chromosome number showed little variation.

Chromosome analysis of +/*an* animals shows that the chromosome 4 carrying the + allele and the chromosome 4 carrying the *an* allele are not visibly different (Fig 4). Thus, if the *an* mutation is a deletion,

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Table 2. Chromosome Number in Metaphases From Cultured Bone Marrow and Kidney

Mouse				Number of Chromosomes†					Percentage of Abnormal Metaphases
Sex	Age (mo)	Genotype	Cultured Cells*	39	40	41	42–50	51–60	
M	4	+/+	BM	3	68				0
			K		75				0
M	6	+/+	BM	1	61				0
			K		40	1			2.4
M	4	*an/an*	BM	15	126	3	3	1	4.7
			K	21	64	5	1	2	8.6
M	4	*an/an*	BM	8	97	1			0.9
			K	7	34	2			4.7
M	6	*an/an*	BM	9	111	3		1	3.2
			K	2	31	4	3	1	19.5
M	6	*an/an*	BM	17	138	1	3		2.5
			K	3	35	1	3		9.5

*BM, bone marrow; K, kidney.

†Metaphases with more than 80 chromosomes or less than 39 chromosomes were disregarded. Nonpolyploid cells with more than 40 chromosomes were counted as abnormal. Cells with 39 chromosomes were considered "normal" with artifactual chromosome loss. By disregarding metaphase spreads with small numbers of chromosomes and counting 39 chromosomes as "normal," the percentage of abnormal metaphases is underestimated.

it spans a region of chromosome 4 too small to be detected by available cytologic methods.

DISCUSSION

We report here that a proportion (5% to 15%) of the mitotic figures from *an/an* mice have an abnormal chromosome number. Hyperploid mitotic figures with more than 40, but less than 80, chromosomes were found in mice of all ages examined, from 14- to 15-day fetuses through 18-month-old adult *an/an* mice. No consistent pattern of extra or missing chromosomes was observed in these cells. Numerical chromosome abnormalities may affect the viability or proliferative capacity of cells. In addition, we found no evidence that the *an* mutation is a deletion, based on cytogenetic observations from banded metaphase spreads of +/*an* mice.

These observations suggest a possible mechanism for the symptoms expressed in Hertwig's anemia mice. Mice of the *an/an* genotype could have an initially normal number of proliferating pluripotential hemopoietic stem cells. An occasional abnormal cell division throughout the cascade of hemopoietic precursor stages could result in an increasingly small population of normal cells. Thus, as abnormal cells are produced and probably die, stem cell populations as measured by CFU-S, BFU-E, and CFU-E would be increasingly depressed. The end result would be an anemia characterized by decreased red cell numbers. Loss of cells from CFU-GM (colony forming units—granulocytic,

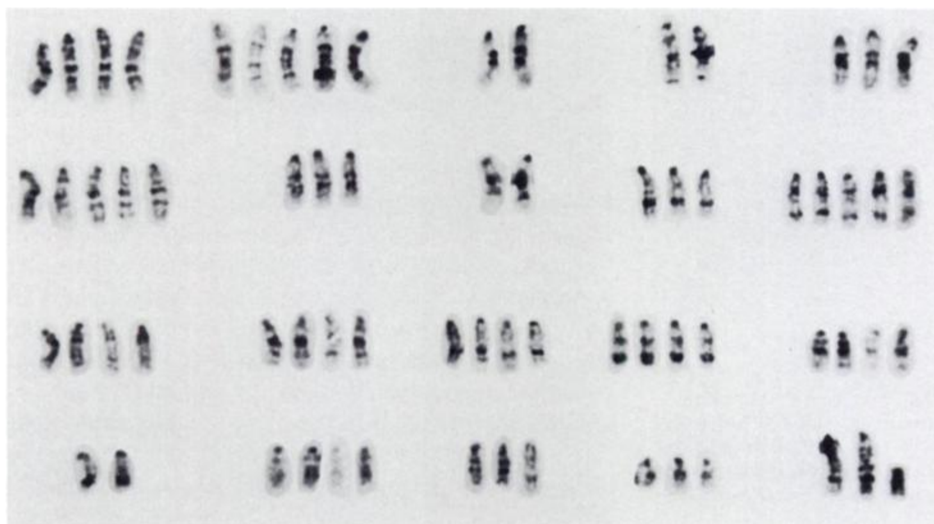

Fig 2. Karyotype of abnormal mitotic figure (69 chromosomes) from *an/an* cultured kidney cell. There is one extra copy of chromosomes 5, 7, 9, 18, 19, and *X*. There are two extra copies of chromosomes 1, 11 through 15, and 17. There are three extra copies of chromosomes 2, 6, and 10.

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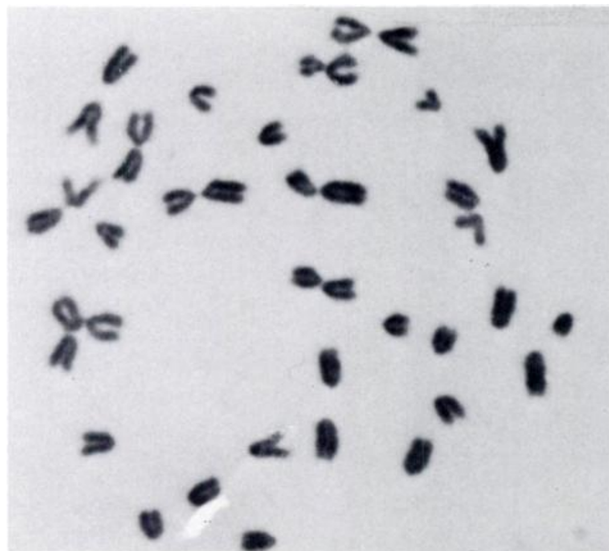

Fig 3. Metaphase spread from an *an/an* cell showing chromosome association.

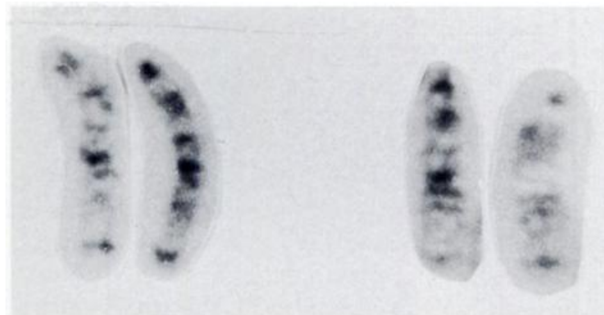

Fig 4. Two pairs of chromosome 4 from two different +/*an* mice. There is no discernible difference in the banding pattern of the chromosome carrying the + allele and that carrying the *an* allele. If the *an* mutation is a deletion, it is too small to be detected by available cytologic methods.

monocytic) populations during maturation could explain the reduction of white blood cell numbers. In addition, such a mechanism could produce the abnormal mitotic figures and degenerating germ cells seen in fetal gonads[3] and result in reduced fertility.

A number of human genetic diseases are characterized by a tendency toward chromosome abnormalities in some cell populations. Although none of the human genetic disease syndromes thus far described seems to be precisely analogous to Hertwig's anemia, there are some parallels. Among the human syndromes with chromosome abnormalities are Fanconi's anemia, in which chromosome breakage occurs[12] rather than anomalous chromosome distribution during mitosis; Friedreich's ataxia, which is characterized by mutagen hypersensitivity and an abnormally high level of background chromosomal aberrations in leukocytes (2.4% compared with 0.4% in controls)[13]; and Roberts' syndrome, which displays abnormal chromosome behavior, chromosome distortion, puffing at the centromere, and abnormal chromatid separation at metaphase.[14]

Table 3. Chromosome Number in Metaphases From Fetal Liver

Mouse Sex	Genotype	Number of Chromosomes* 39	40	41-50	51-60	61-79	Percentage of Abnormal Metaphases
F	+/*an*	2	19				0
M	+/*an*		30				0
M	+/*an*		42				0
F	+/*an*		52				0
F	+/*an*		52				0
M	+/*an*	2	50				0
M	*an/an*	3	39	2	1		6.7
F	*an/an*	1	41	2		1	6.7
M	*an/an*	1	53	3		1	6.9
M	*an/an*	2	49	3	1	2	10.5

*Metaphases with more than 80 chromosomes or less than 39 chromosomes were disregarded. Nonpolyploid cells with more than 40 chromosomes were counted as abnormal. Cells with 39 chromosomes were considered "normal" with artifactual chromosome loss. By disregarding metaphase spreads with small numbers of chromosomes and counting 39 chromosomes as "normal," the percentage of abnormal metaphases is underestimated.

Some interesting observations on chromosome segregation and centromere separation may be relevant to the Hertwig's anemic mouse. Vig[15,16] reported that centromeres separate at anaphase in a nonrandom order in humans, mice, *Vicia faba,* and *Crepis capillaris* and suggested that such sequential centromere separation patterns may be a feature of all eukaryotes. Verma et al[17] found that acrocentric human chromosomes with active (Ag-positive staining) nucleolus organizer regions are preferentially associated at mitosis. Furthermore, they reported that in three trisomy 21 patients, each chromosome 21 was Ag-positive staining. Both of these reports lead one to speculate that chromosome association and the sequence of centromere separation may be related to nondisjunction and the production of aneuploid offspring.

Stallard et al[18] observed that normal parents of aneuploid offspring have a fivefold higher frequency of one or more extra chromosomes in metaphase spreads from their peripheral leukocytes (0.64% *v* 0.12% for aged-matched controls). The extra chromosome in any given cell may not be the same as the extra chromosome from another cell nor the same as the aneuploid chromosome present in the offspring. Stallard et al[18] speculated that this may indicate a chromosomal nondisjunction tendency and provide a way of detecting parents at risk for producing aneuploids.

Staessen et al[19] obtained similar results in studies of parents with recurrent spontaneous abortions or with a

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trisomic child—lymphocytes of these parents had a significantly greater number of hyperploid cells than controls. Thus, nondisjunction during gamete production appears to be correlated with abnormal cell division in a proliferating somatic cell population.

Hertwig's anemic mice have a mild anemia and reduced fertility, both probably resulting from loss of abnormal cells. Observations reported here indicate that a proportion of somatic cells undergo abnormal cell divisions and produce cells with abnormal chromosome numbers. Abnormal mitotic figures have also been observed in fetal gonads.[3] It is possible that some biochemical defect exists in Hertwig's anemic mice that upsets the normal cell division process and has far-reaching physiologic effects.

Another feature of Hertwig's anemia that may be related to the chromosomal abnormality is the high incidence of reticulum cell neoplasia, type A (histiocytic sarcoma). This mouse may prove to be a good model for a human neoplasm and may reveal some specific chromosomal change that promotes the development of this tumor.

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