

Brief Report

DISPUTED MATERNITY LEADING TO IDENTIFICATION OF TETRAGAMETIC CHIMERISM

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CHIMERISM, the presence of two genetically distinct cell lines in an organism, either is acquired through the infusion of allogeneic hematopoietic cells during transplantation¹ or trans-fusion² or is inherited. In fraternal twins, chimerism occurs by means of blood-vessel anastomoses. A less common cause of congenital chimerism — so-called tetragametic chimerism — occurs through the fertilization of two ova by two spermatozoa, followed by the fusion of the zygotes and the development of an organism with intermingled cell lines.³ Examples have been found in mice⁴ and other mammalian species,⁵⁻⁷ including humans.⁸⁻¹⁷ Affected persons are identified by the finding of two populations of red cells⁹ or ambiguous genitalia and hermaphroditism,^{11,15,16} alone or in combination; such persons sometimes also have patchy skin or eye pigmentation.¹⁷

We describe a phenotypically normal woman in whom tetragametic chimerism was unexpectedly identified after histocompatibility testing of family members suggested that she was not the biologic mother of two of her three children.

CASE REPORT

A 52-year-old woman had renal failure as a result of focal sclerosing glomerulonephritis. In preparation for kidney transplantation, the patient and her immediate family underwent histocompatibility testing (Fig. 1A). The results suggested that the patient could not

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be the biologic mother of two of her three sons, who had her husband's HLA haplotype and a unique collection of HLA determinants, instead of one of the expected maternal haplotypes (Fig. 1).

On examination, she was a phenotypically normal female without abnormal pigmentation of the skin or eyes. Her birth had been unremarkable. Additional laboratory investigations were performed, with the patient's written informed consent.

METHODS

Tissue Collection

Samples of buccal mucosa, hair follicles, and skin were obtained; samples of formalin-fixed thyroid tissue were obtained from a previously excised benign thyroid nodule; and bladder tissue was obtained during cystoscopy. Epidermal keratinocytes and fibroblasts were isolated from bladder-biopsy specimens, and skin-fibroblast cultures were also established, as described previously.¹⁸

Blood Grouping and HLA Studies

Tube-based serologic testing was used to type red cells for ABO and other blood-group antigens.¹⁹ Blood samples were used for the serologic and molecular typing of HLA class I markers; class II typing was performed with the use of molecular methods alone. Tissue samples, either without further modification or after culture, in the case of bladder and skin specimens, were used to extract DNA (QIAAMP Tissue Kit, Qiagen) for molecular typing of HLA class I and class II markers. Molecular typing was performed with the use of the polymerase chain reaction (PCR), sequence-specific primer amplification,^{20,21} and published primer sequences²² and with the use of PCR and sequence-specific oligonucleotide probes (HLA Quick-Type kits, Lifecodes), according to previously described amplification conditions.²³ To increase the sensitivity of haplotype detection, we also used nested PCR amplification: the initial round of amplification consisted of 30 cycles; 10 μ l of the amplification product was then removed and used as a template for another 30 cycles.²⁴ Haplotypes were assigned on the basis of allele data obtained from studies of the patient and her family.

Cytogenetic Analysis

Chromosomes were prepared from cultured skin fibroblasts and phytohemagglutinin-stimulated lymphocytes in prometaphase and metaphase and stained according to standard protocols.^{25,26} To rule out low-level trisomy or tetrasomy, in situ hybridization of cells in interphase was performed as previously described, with the use of a pericentromeric sequence for chromosome 6 (D6Z1).²⁷

Determination of Sex Chromosomes

The amelogenin gene, present on both X and Y chromosomes, was amplified by PCR (GenePrint STR systems, Promega) according to the manufacturer's recommendations. XX chromosomes have a single 212-bp fragment; XY chromosomes have both 212-bp and 218-bp fragments.

Short Tandem-Repeat Microsatellite Markers

We analyzed the number of repeats of small (dinucleotide, trinucleotide, or tetranucleotide) motifs in a given region of a chromosome to identify genetic polymorphisms. We studied 22 short tandem repeats on 16 autosomes and the X chromosome. We used commercially available kits for the following loci: TPOX, D3S1358, FGA, D8S1179, THO1, vWA, Penta E, D18S51, and D21S11 (Powerplex 2.1 GenePrint STR systems, Promega); D16S539, D7S820, D13S317, and D5S818 (GammaStar, GenePrint STR systems); FGA, D7S820, D1S533, and D9S304 (Multiplex II, Lifecodes); and D12S1090, D3S1744, and D18S849 (Multiplex I, Lifecodes).²⁸⁻³⁰ Alleles were designated according to the recommen-

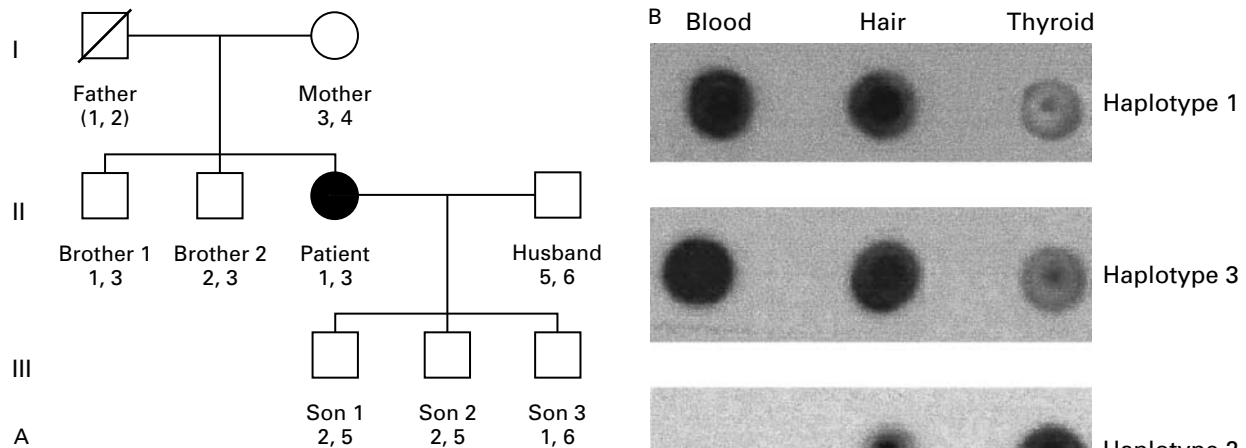


Figure 1. Pedigree and Results of HLA Haplotyping of Blood Samples from the Patient and Her Family (Panel A) and of the Samples of Blood, Hair Follicle, and Thyroid from the Patient (Panel B).

The proband's father was deceased, and his haplotypes, shown in parentheses in Panel A, were deduced from studies of the other family members. Haplotype 1 was HLA-A*66,B*41,DRB1*04,DQB1*0301; haplotype 2 was HLA-A*25,B*08,DRB1*08,DQB1*04; haplotype 3 was HLA-A*02,B*40,DRB1*04,DQB1*0301; haplotype 4 was HLA-A*11,B*27,DRB1*0101,DQB1*0501; haplotype 5 was HLA-A*03,B*35,DRB1*0101,DQB1*0501; and haplotype 6 was HLA-A*24,B*15,DRB1*04,DQB1*0302. In Panel B, polymerase chain reaction and sequence-specific oligonucleotide-probe hybridization were used for haplotyping. The probe sequences included 5'GAGACGGCCCATGAGGCG3' in the case of HLA-A*66, 5'TATTGGGACGGGAGACA3' in the case of HLA-A*02, 5'GAGGTATTTCGACACCGCC3' in the case of HLA-B*08, and 5'ATCTGCAAGGCCAAGGCA3' in the case of HLA-B*27. Only haplotypes 1 and 3 were evident in the blood sample. On the basis of a visual analysis of the signal strength, hair-follicle samples showed a preponderance of DNA associated with haplotypes 1 and 3 and a smaller amount of DNA associated with haplotypes 2 and 4. In contrast, thyroid-tissue samples had a preponderance of haplotypes 2 and 4 and smaller amounts of haplotypes 1 and 3.

dations of the DNA Commission of the International Society for Forensic Haemogenetics; size ladders were provided by the various manufacturers.³¹ We also amplified DNA using radioactively end-labeled primers for D2S160, D2S2216, D20S195, and DXS1073 (GIBCO-BRL, Life Technologies). PCR products were separated by polyacrylamide-gel electrophoresis and identified by autoradiography.³²

Mixed-Lymphocyte Culture and Cell-Mediated Lysis

The mixed-lymphocyte culture detects mismatched major-histocompatibility-complex (MHC) class II antigens (HLA-DR and DQ alleles) on the surface of a person's irradiated lymphocytes and monocytes (stimulator cells) by using as an end point the degree of proliferation of another person's CD4 (responder) cells.³³ In this study, the proband was the source of the responder cells, and stimulator cells were obtained from family members and from four normal subjects used as controls. Stimulator and responder cells were cocultured for six days, the wells were labeled with tritiated

thymidine, and the degree of proliferation of CD4 cells was determined. The result was expressed as the relative response, defined as the ratio of thymidine uptake by the responder cells in response to exposure to the irradiated stimulator cells, as compared with the exposure to control cells.

Cell-mediated lysis is used to assess the capacity of CD8 lymphocytes to kill cells that are mismatched for MHC class I antigens (HLA-A, B, and C alleles).³³ In this procedure, cells from the proband were cultured with irradiated target cells to create primed effector cells. Chromium-51-labeled target cells from various sources were then added to the effector cells at various ratios of effector to target cells. After a four-hour incubation, the supernatants were removed and analyzed. The result was expressed as the percentage of specific cytotoxicity, defined as the amount of chromium-51 released in comparison to the total cell-associated chromium-51.

RESULTS

Blood Typing

The patient's red cells were group A, Rh-positive; antibody against group B (agglutination titer, 3+) was present in her plasma. Her husband's red cells were blood group O, and the two sons of questionable maternity were group A and group O. Her red-cell phenotype was R₁r(Cde/cde),K⁻,Fy(a+b+),Le(a-b+),P₁⁻,M+N+S+s+s+, and there was no evidence of two distinct cell populations.

HLA Studies

Haplotyping showed that one of the patient's brothers had a haplotype of HLA-A*25,B*08,DRD1*-

TABLE 1. MICROSATELLITE ANALYSIS OF DNA FROM VARIOUS TISSUES FROM THE PATIENT AND OF BLOOD FROM FAMILY MEMBERS.*

CHROMOSOME NO. AND LOCUS	VARIABLE	TYPE AND SOURCE OF SAMPLE												
		BLOOD, PATIENT	HAIR, PATIENT	THYROID, PATIENT	BUCCAL MUCOSA, PATIENT	SKIN, PATIENT	BLOOD, SON 1	BLOOD, SON 2	BLOOD, SON 3	BLOOD, FATHER†	BLOOD, MOTHER	BLOOD, BROTHER 1	BLOOD, BROTHER 2	
1, D1S533	No. of repeats	15/11	15/11/(8)	15/11/(8)	15/11	15/11	15/9	9/8	ND	8/11	15/14	14/8	15/11	
2, TPOX	No. of repeats	11/8	11/8	11/8	11/8	11/8	11/11	12/11	ND	8/11	11/11	11/8	11/11	
2, D2S160	Size of allele (bp)	217/219	ND	ND	ND	213/217	213/215	ND	ND	ND	ND	217/219	217/219	
2, D2S2216	Size of allele (bp)	211/211	ND	ND	ND	215/221	215/221	ND	ND	ND	ND	211/221	211/221	
3, D3S1358	No. of repeats	17/14	17/14	17/(15)/(14)	17/14	17/14	15/15	17/15	ND	14/18	17/15	17/15	18/15	
3, D3S1744	No. of repeats	20/17	20/(19)/17	20/19/(17)	20/(19)/17	20/(19)/17	19/18	20/18	18/17	17/19	20/20	20/17	20/17	
4, FGA	No. of repeats	22/20	(23)/22/20	23/22/(20)	22/20	(23)/22/20	22/20	22/20	ND	ND	23/20	23/22	23/22	
5, D5S818	No. of repeats	12/11	12/11	12/11	12/11	12/11	12/11	11/11	11/11	ND	11/11	12/11	12/11	
7, D7S820	No. of repeats	10/10	10/(8)	10/(8)	10/10	10/10	11/8	11/8	11/10	10/12	10/8	10/10	12/8	
8, D8S1179	No. of repeats	16/13	16/13/(11)	16/11/(13)	16/13	16/13	16/13	16/11	ND	13/16	13/11	16/11	16/11	
9, D9S304	No. of repeats	12/4	12/(9)/4	9/4	12/(9)/4	12/4	12/4	12/9	ND	9/12	4/4	9/4	9/4	
11, THO1	No. of repeats	9.3/9	9.3/9	9.3/(9)	9.3/9	9.3/9	9.3/9.3	9.3/9.3	ND	10/13	9.3/9	10/9.3	9.3/9.3	
12, vWA	No. of repeats	17/14	17/14	17/14	17/14	17/14	17/17	19/17	ND	ND	17/14	17/14	18/14	
12, D12S1090	No. of repeats	23/20	23/(22)/20	22/22	23/(22)/20	23/(22)/20	24/22	24/22	24/23	22/23	22/20	22/22	22/22	
13, D13S317	No. of repeats	11/8	(13)/11/8	13/11/(8)	11/8	11/8	13/11	11/11	11/11	8/11	13/11	11/11	11/11	
15, Penta E	No. of repeats	15/14	15/14/(13)	14/13	15/14	15/14	17/14	13/5	ND	13/14	15/14	14/13	14/14	
16, D16S839	No. of repeats	13/9	13/(11)/9	(13)/11/9	13/9	13/9	12/11	11/9	13/11	9/11	13/11	11/9	13/11	
18, D18S51	No. of repeats	14/11	14/11	11/11	(14)/(11)	14/11	13/11	19/11	ND	11/14	14/11	14/14	11/11	
18, D18S49	No. of repeats	18/16	18/16	18/17	18/16	18/16	18/16	17/17	18/17	ND	18/17	17/16	17/16	
20, D20S195	Size of allele (bp)	139/149	ND	ND	ND	139/147	139/147	ND	ND	ND	ND	139/149	139/149	
21, D21S11	No. of repeats	31/28	31/28	31/30.2	31/28	31/28	31/29	34.2/31	ND	ND	31/30.2	31/30.2	31/28	
X, DXS1073	Size of allele (bp)	311/313	ND	ND	ND	313/313	311	ND	ND	ND	ND	313	313	

*Values in parentheses refer to minor-intensity alleles (for which the height of the curve was less than 50 percent of the height of the curves for other alleles at the same locus). Discrepancies in results between blood samples and tissue samples from the patient are italicized. Results in two of the patient's sons that appeared to rule her out as their mother are shown in boldface type. ND denotes not done. †Because samples were not available from the patient's father, the results were deduced from studies of other family members.

08,DQB1*04, which was presumably paternally inherited. PCR and sequence-specific oligonucleotide-probe hybridization showed four haplotypes in samples of skin, thyroid, bladder epithelial cells, bladder fibroblasts, buccal mucosa, and hair-follicle cells from the patient but only two haplotypes in her blood. In tissues with four haplotypes, one of two pairs always predominated, either haplotype 1 and haplotype 3 or haplotype 2 and haplotype 4 (Fig. 1B).

Cytogenetic Analysis

Cytogenetic analysis of both blood and cultured skin fibroblasts from the patient demonstrated a normal karyotype of 46,XX. An analysis in which the amelogenin gene was used as a marker showed a female sex chromosome complement. Using fluorescence in situ hybridization, we examined 200 nuclei to determine the number of copies of chromosome

6 in each nucleus. All 200 had a normal diploid complement.

Short Tandem-Repeat Microsatellite Markers

Microsatellite analysis of DNA from various tissues from the patient and her family identified more than two alleles at one or more loci in 14 of the 17 chromosomes from the patient that were studied (Table 1).

Mixed-Lymphocyte Culture and Cell-Mediated Lysis

In the mixed-lymphocyte culture, the patient's lymphocytes had no proliferative activity against cells from her HLA-identical brother (Brother 1, who had haplotypes 1 and 3), her haploidentical brother (Brother 2, who had haplotypes 2 and 3), or her haploidentical mother (haplotypes 3 and 4). However, the patient's lymphocytes responded appropriately to lym-

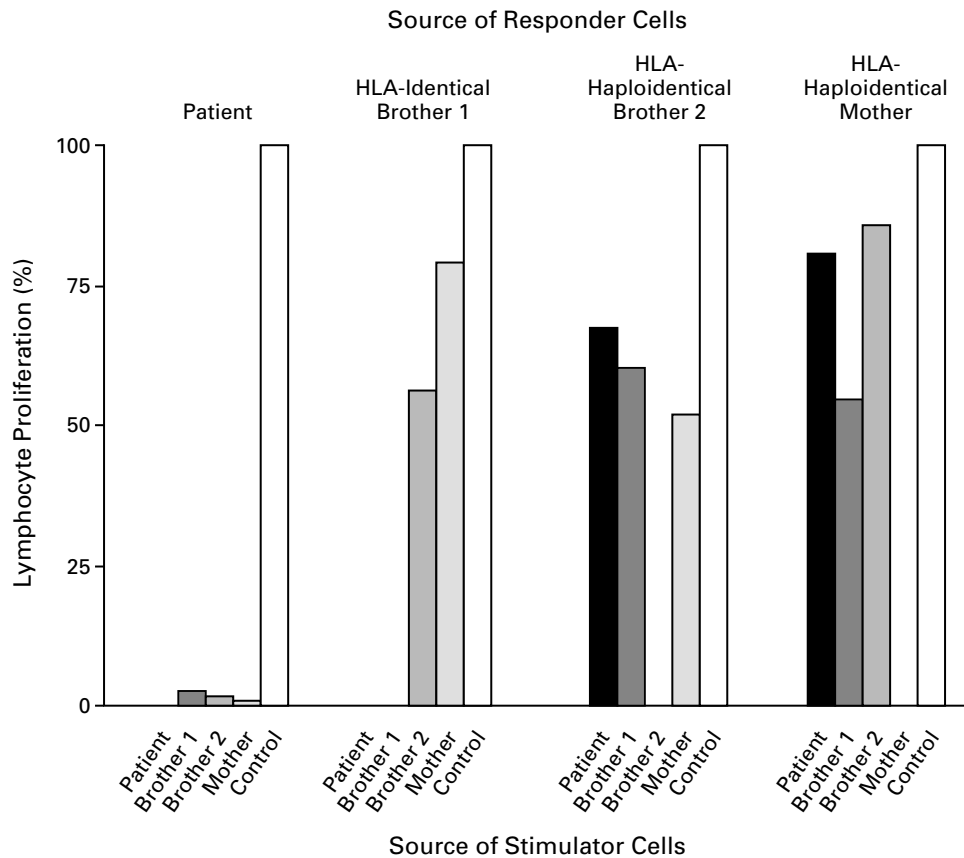


Figure 2. Mixed-Lymphocyte Cultures of Cells from the Patient, Her Two Brothers, and Her Mother and Pooled Cells from Unrelated Control Subjects.

Lymphocyte proliferation is reported as a relative response and represents the uptake of tritiated thymidine by responder cells in response to an irradiated population of stimulator lymphocytes, as compared with the uptake of thymidine in response to irradiated control lymphocytes.

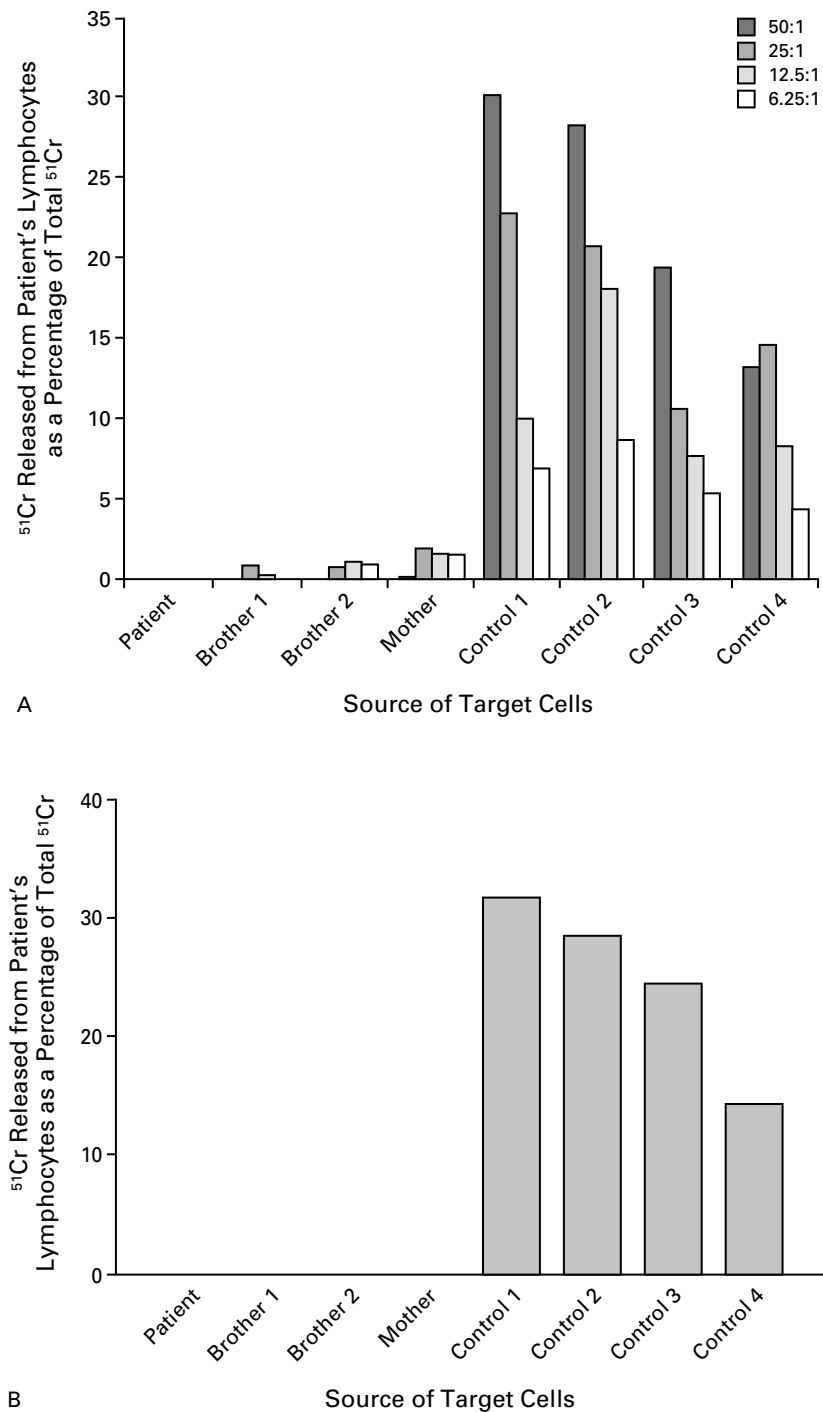
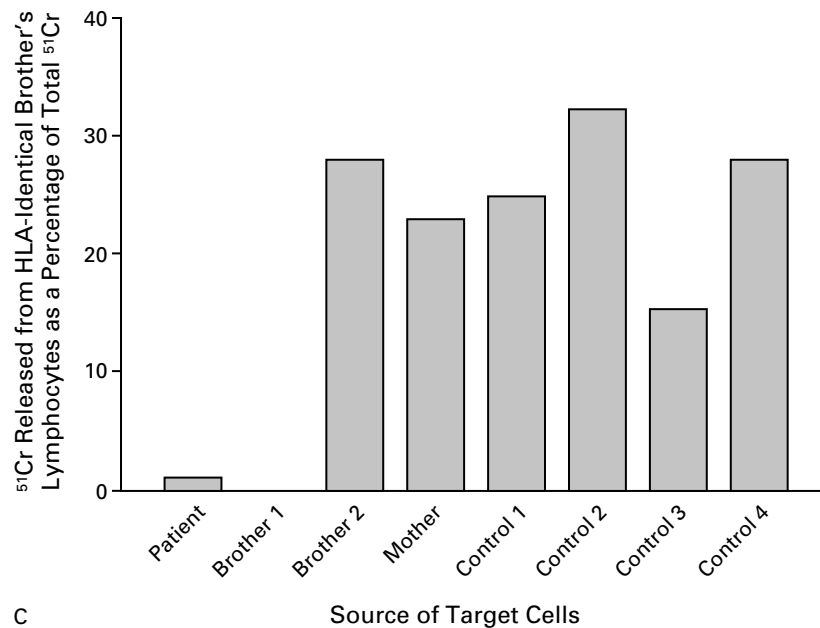


Figure 3. Cell-Mediated Lysis.

Effector cells consisted of primed lymphocytes from the patient, and target cells consisted of phytohemagglutinin-stimulated, irradiated ^{51}Cr -labeled lymphocytes from the patient (as an autologous negative control), her HLA-identical brother (Brother 1), her haploidentical brother (Brother 2), her haploidentical mother, and four unrelated controls. In Panel A, four different effector:target ratios were evaluated (50:1, 25:1, 12.5:1, and 6.25:1), and the percentage of ^{51}Cr released in relation to total amount of cell-associated ^{51}Cr was calculated. At an effector:target ratio of 50:1, the patient's cells were unable to kill cells from her HLA-identical brother, her HLA-haploidentical brother, or her mother (Panel B), whereas cells from her HLA-identical sibling B1 (Brother 1) were able to lyse cells from both his mother and his brother (Panel C, next page).



C

Source of Target Cells

phocytes from unrelated control subjects (Fig. 2). Her HLA-identical brother had normal proliferative responses to all cells except those from the patient, and her haploidentical brother and mother had proliferative responses to cells from all family members and the control subjects. In studies of cell-mediated lysis, the patient's cells were unable to kill the cells from her brothers or mother, regardless of the effector:target ratio used (Fig. 3A and 3B), but they did lyse lymphocytes from the four unrelated controls. Cells from her HLA-identical brother lysed cells from both his brother and his mother (Fig. 3C).

DISCUSSION

This case represents an unusual example of tetragametic chimerism in a phenotypically normal, fertile XX/XX female who had no evidence of chimerism in peripheral blood. Figure 4 outlines the probable cause of this chimerism: separately fertilized XX zygotes, one with HLA haplotypes 1 and 3 and the other with haplotypes 2 and 4, are thought to have fused early in development. The distribution of cell lines varied in individual tissues, except in blood, which appeared to be derived from only one cell line, bearing HLA haplotypes 1 and 3. It is highly unlikely that the levels of the second cell line were below the limits of detection of our assays; we used sensitive techniques and multiple informative probes, which we have shown can identify as few as 1 in 100,000 cells in ex-

perimental mixes of two cell populations (unpublished data). Because of the single cell line in our patient's blood, blood-based studies of blood groups,¹⁴ molecular HLA typing,³⁴ and DNA polymorphism analysis,¹⁰ which have all been used to identify chimeras, were not informative.

We are aware of only two other possible cases of human tetragametic chimeras with single cell lines in blood. In one case, discrepancies in the blood type between a woman and her children suggested that she was not the biologic mother.³⁵ As in our patient, the children's nonpaternal haplotype was identified in maternal grandparents. However, the authors estimated that they would not have been able to detect a population of cells that was less than 0.5 percent of peripheral-blood cells.³⁶ In another patient, a phenotypically normal man whose red cells were blood group B, chimerism was detected because of a surprisingly weak titer of antibody against group A and small amounts of group A substance on his red cells. The patient proved to be an XX/XY chimera with only XY lymphocytes in his blood. The XY line produced group B red cells; the XX line encoded a group A glycosyl transferase. The activity of this enzyme in nonhematopoietic XX tissues resulted in group A substance that was passively adsorbed by the patient's XY group B red cells.⁹

In a mouse model of tetragametic chimerism, in which blastomeres from two embryos were cocultured

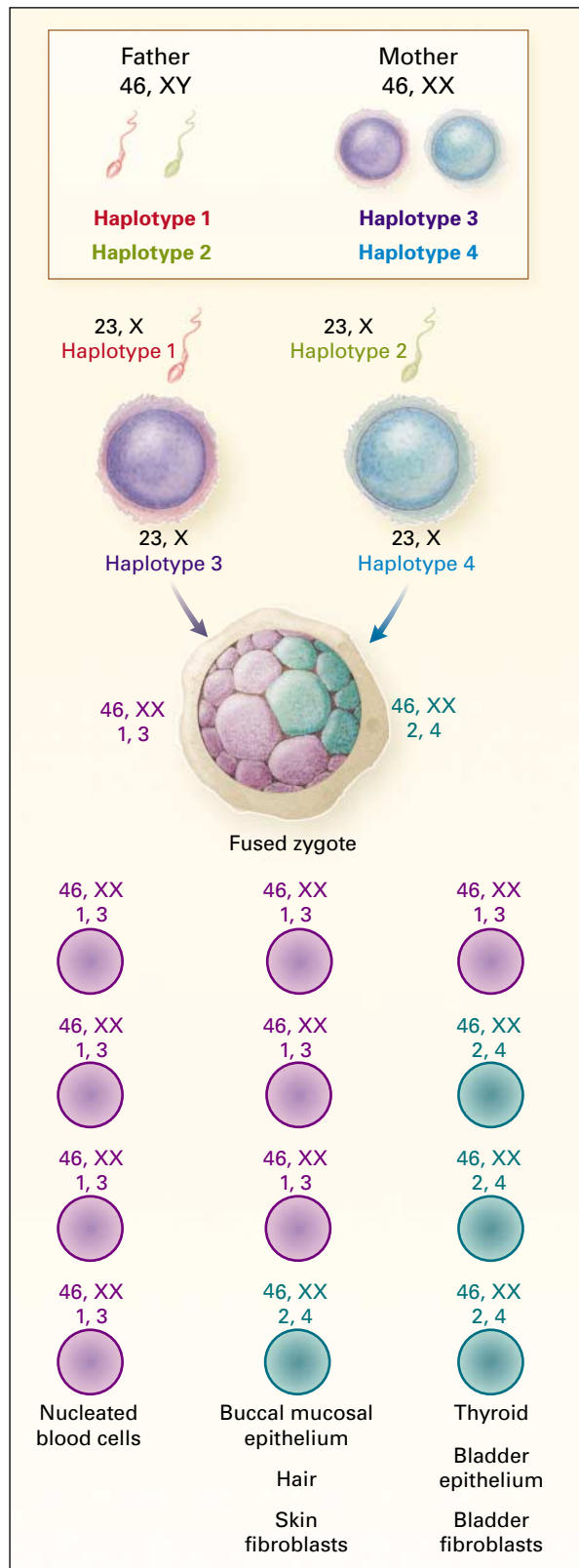


Figure 4. Proposed Derivation of Various Tissues in the Patient. The findings were based on the results of polymerase-chain-reaction analysis. Both cell lines are represented to some extent in all tissues except blood.

to form a chimera, 12 of 34 such mice had only one red-cell population in the blood even though they had two cell lines in other tissues.⁴ This finding could be consistent with the presence of a single cell line of clonal origin beginning early in development.³⁷ Alternatively, a selective advantage could have caused one clone to be selected early in life. The latter possibility is supported by the finding, in a study of tetragametic rams, that one of the two red-cell lines completely disappeared over a period of five years in two of four chimeric animals.⁵

Because of the apparent rarity of tetragametic chimerism and the importance of the use of molecular techniques to confirm its presence, this condition may be underdiagnosed. Furthermore, if a single cell line predominates in the blood, the chimeric state may not be detected unless family studies are undertaken. Even then, the findings may be misinterpreted as ruling out maternity or paternity. Molecular studies of other tissues for chimerism should be considered in such cases. Furthermore, the need to consider this diagnosis may be increasingly relevant: in vitro fertilization is associated with a 33-fold increase in twinning¹⁶ and an increased incidence of tetragametic chimerism, possibly because the embryos are in close contact and fuse before they are implanted¹⁶ or because of double fertilization of an ovum with two nuclei.^{38,39}

Finally, the tetragametic state has important implications for organ or stem-cell transplantation. Chimeras typically have immunologic tolerance to both cell lines. Even though our patient had only one cell line in her blood, her T lymphocytes did not respond to cells from family members with any combination of the four familial HLA haplotypes. These results are consistent with those of studies of tetragametic mice with single red-cell populations, which also demonstrated tolerance to skin grafts from parental strains.⁴ Thus, for a tetragametic human, a wider array of relatives (including, in our patient, all her children) and other persons may be eligible to be organ donors.

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