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Monozygotic twins from in vitro fertilization*

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A case of identical twins following in vitro fertilization and embryo transfer (IVF-ET) is described. Two embryos were transferred, but it is apparent that only one implanted and subsequently divided in the early implantation phase to produce identical male twins within a monochorionic, diamniotic placental and membrane configuration. Additional marker studies provide an overall probability of < 0.001 for dizygosity. There is unlikely to be any relationship between this event and the technique of IVF-ET. Fertil Steril 41:833, 1984

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Since the birth of the first child conceived by in vitro fertilization and embryo transfer (IVF-ET) in July 1978,[1] a number of medical teams throughout the world have reported similar achievements, and it is likely that the number of infants now delivered from this technique exceeds 200. The first such child born in Western Australia is a healthy male who was delivered on July 13, 1982.[2] Although Steptoe and Edwards[1] achieved their first success in a natural cycle with fertilization of a single preovulatory oocyte and the transfer of a single 8-cell embryo, most teams are now reporting their results from stimulated cycles with the development and subsequent transfer of several embryos. This has led to a consequent improvement in the pregnancy rate per laparoscopy, but several groups have now reported twin gestations, and recently healthy triplets were delivered in South Australia following

multiple ET.[3] To date we have delivered 21 healthy infants following IVF-ET. Three multiple gestations (two sets of twins and one set of triplets) arose following multiple transfers, and this report presents details of an unexpected outcome from one of the sets of twins.

MATERIALS AND METHODS

In the initial phase of the IVF program, couples were selected strictly on the basis of nonpatent or absent fallopian tubes. The patient was a 30-year-old dental nurse and her husband a 32-year-old dental accountant. They are both of English background and were drawn from a series of cases undergoing IVF because of nonpatent or absent fallopian tubes. The couple have been married for 9 years, with primary infertility of 5 years' duration. Investigations revealed a left distal occlusion from hydrosalpinx and a right proximal occlusion from an isthmic nodule. Subsequently, microsurgery was undertaken with a salpingostomy on the left fallopian tube and a resection/reanastomosis on the right side. Histology disclosed that the nodular lesion was an endometrioma. However, pregnancy failed to ensue; and a review hysterosalpingogram 9 months later indicated bilateral tubal occlusion similar to the preoperative state. The patient was subsequently admitted into the IVF program.

The first attempt was undertaken in August 1981 during the early establishment phase of this program. Following clomiphene stimulation (150 mg daily for 5 days) from days 2 to 6 of the cycle, two preovulatory oocytes were aspirated on day 14. Unfortunately, fertilization failed to ensue, and this failure was attributed to a technical problem within the laboratory leading to heat damage of the oocytes. A second treatment cycle was undertaken in August 1982. Again the cycle was stimulated with clomiphene, 150 mg on days 2 to 6 of the cycle, and ovulation was triggered with human chorionic gonadotropin, 5000 U intramuscularly. Laparoscopy was carried out 35 hours later, on day 15 of the cycle, and two mature preovulatory oocytes were obtained. Four hours later the husband provided his semen. After centrifuging the specimen twice and replacing the seminal plasma with fertilizing medium (modified Tyrode's solution),[4] an overlay technique was used to provide a highly motile sperm preparation. Approximately 200,000 motile sperm were added to each tube containing the oocyte in 1 ml of fertilizing medium with added 7.5% deactivated maternal serum. The oocytes were dissected out of their coronal coats 16 hours later, when two pronuclei were recognized in each oocyte. The embryos were further cultured in the modified Tyrode's solution containing 15% deactivated maternal serum. At 42 hours the embryos were transferred via the cervix with the patient in lithotomy position and 20 degrees head-down tilt. One was a slightly fragmented 2-cell embryo, and the other was at the 4-cell stage, with discrete blastomeres. The embryos were transferred by a double catheter technique: the outer Teflon tube transgressed the cervical canal 4 cm, and the inner Teflon tube, with an outer diameter of 1.2 mm, entered the uterine cavity 6 cm from the external os. This was known from a previous uterine sounding to be 1 cm short of the fundus, and this measurement was confirmed during the treatment cycle by ultrasonography.

RESULTS

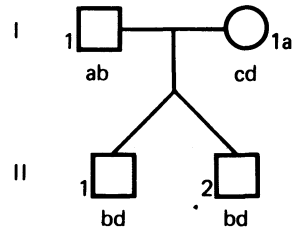
Pregnancy was diagnosed when serum β-human chorionic gonadotropin assays on days 10, 13, and 16 detected levels of 13, 33, and 100 U/l, respectively. The nonpregnant levels are < 4 U/l. Seven weeks after ET, an ultrasonic examination detected twin gestational sacs in the uterus, each containing an embryo with a definite heartbeat. Subsequently, the pregnancy proceeded uneventfully with normal fetal growth monitored every 6 weeks on ultrasound examination of the biparietal diameter and abdominal circumference. In the third trimester both twins presented as persisting breeches, and hence elective cesarean section was undertaken 10 days prior to term, on May 12, 1983, under epidural anesthesia. The twins were healthy, lusty male infants, the first weighing 3050 gm, and the second, 2680 gm. They appeared remarkably similar, despite the weight difference.

Table 1. *Blood Groups—Relative Probability of Dizygosity*[a]

B	0.4741
N_sN_s	0.4827
R_1R_1	0.5021
P_1+	0.8489
k/k	0.9485
$Fy^{a\ neg}$	0.6319
$Le^{a\ neg}$	0.8681

[a]Blood group studies show the chance of dizygosity in the twin pair to be 0.0507, indicating a high relative probability of monozygous twinning.

FAMILY TREE WITH HAPLOTYPE ASSIGNMENTS



Haplotypes *

a = A 1 B 7
b = A19 B40
c = A 1 B17
d = A 2 B27

Figure 1
HLA phenotypes and genotypes. HLA phenotypes: father, (I1) HLA-A1, 19, B7, 40; mother, (I1a) HLA-A1, 2, B17, 27; first twin, (II1) HLA-A2, 19, B27, 40; second twin, (II2) HLA-A2, 19, B27, 40. Because the HLA-A and B loci are closely linked, alleles at these loci are inherited "en bloc" as maternal or paternal haplotypes.

There was a single placenta located over the anterior fundal position. It measured 25 × 21 × 3 cm. The membrane structure revealed a single chorion containing two distinct amniotic sacs. Microscopic examination of the placenta and membranes confirmed that the placenta was normal, with a monochorionic configuration, and the intervening layer between the fetal sacs was composed of two amniotic membranes. Each cord was normal, containing three vessels.

Blood grouping of both infants yielded identical results for the ABO, Rhesus, Kell, MNS, Duffy, Lewis, and P systems; both were B(O), CCDēē, k/k, N_sN_s, Fy (a negative, b positive), Le (a negative, b negative), P_1 positive in type. Table 1 provides the relative probability of dizygosity in the twin pair.[5] Note that only five alleles can be determined with certainty for the Rhesus phenotype, because antibody for the detection of d antigen is not available. The genotype CDe/CDe (R_1R_1) is assumed because the frequency of occurrence of the CDe (R_1) gene complex is 0.476[6]; the only alternative in this instance CDe (R_1) is 0.008 in a British population. The occurrence of the R_1R_1 genotype is at least 20 times > R_1r[1]; and for the purpose of dizygosity probability estimations, the latter genotype is not considered because it reduces the likelihood even further.

At the 6-week examination, the mother stated she had difficulty recognizing one infant from the other, except when they were close together. The infants were fully breast-fed and progressing normally. Twin 1 weighed 4350 gm and had a length of 56 cm and a head circumference of 39 cm. Twin 2 weighed 4050 gm and had a length of 54.5 cm and a head circumference of 38.5 cm. Both infants had a small umbilical hernia but otherwise were physically and neurologically normal.

Subsequently, dermatoglyphic studies were carried out, including palmar crease patterns, finger and hallucal patterns, and a dermal ridge count on the fingers. The palmar crease patterns, although not identical, were very similar for each twin; and although difficulty was encountered in obtaining dermal ridge patterns by inked impressions, the observed sum of ridges counted from the center of a whorl or loop to the farthest triradius revealed a difference of only ten ridges, providing a relative chance for dizygosity as $P = 0.26$.[5]

Human leukocyte antigen (HLA) assignments were analyzed for the parents and twin siblings by the standard National Institutes of Health lymphocyte microtoxicity test for the A an B locus alleles.[7] The phenotypes, family tree, and assigned haplotypes are shown in Figure 1. The twins are phenotypically identical and haploidentical. The probability of haploidentity for dizygotic twins is 0.25.

Cytogenetic analyses of both twins and parents were also performed on 72-hour peripheral blood cultures. Banding techniques designed to reveal centromeric bands and nucleolus organizing regions were used.[8] The only distinctive polymorphism observed was a large heterochromatic re-

Table 2. *Marker Studies—Relative Probability of Dizygosity*

Initial chance	2.333
Likeness in sex	0.5000
Seven blood group studies	0.0507
Ridge count difference	0.26
Chromosome polymorphism	0.25
HLA haploidentity	0.25

$p(D) = P_1(D) \times P_2(D) \ldots\ldots P_n(D)$[a]
$n = 12$
$p(D) = 0.000961$

Therefore, the probability of the pair being dizygotic $= \dfrac{p(D)}{1 + p(D)} = \dfrac{0.000961}{1.000961}$

$= 0.000961$

Applying 12 markers apart from placental and membrane configuration, monozygosity is confirmed with $P < 0.001$.

[a]From Smith and Penrose.[5]

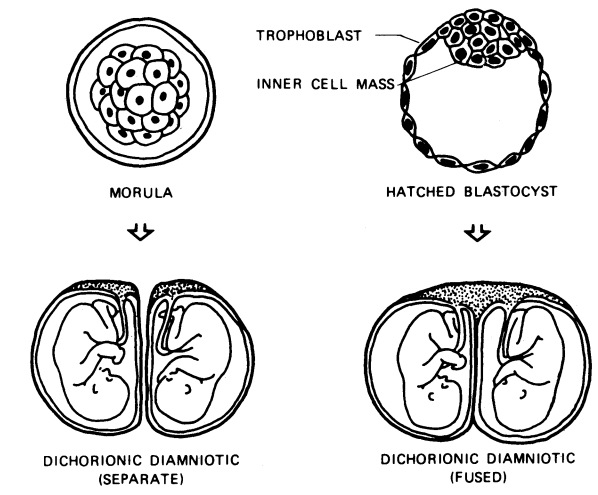


Figure 2
Twinning arising from division in the preimplantation stage of embryo development.

gion in one maternal 9 chromosome, which stained strongly positive using the Giemsa staining technique at pH 11. This polymorphism was not apparent in either twin, and the likelihood of its appearance in sibling pairs is 0.25.

In estimating the probability of dizygosity, p(D), the formula described by Smith and Penrose[5] is applied and depicted in Table 2. Because the parents are of English background, British figures have been applied for the initial relative chance of dizygosity based on the expectation that there are an equal number of like-sexed and unlike-sexed dizygotic twins in a population; in England this is 70:30, or 2.333:1.

DISCUSSION

The natural incidence of twins occurring spontaneously is 1 in 89 deliveries.[9] Approximately 20% of twin gestations are monozygotic, and three distinct types are recognized, depending on the stage at which the embryo splits or reduplicates itself. Whereas this mechanism is quite unknown, Figures 2 and 3 illustrate the presumed embryology of twinning.[10] Early separation of the embryo before day 7 is likely to lead to the complete reduplication of separate amniotic and chorionic membranes, as the amnioblasts are still closely approximated to the cytotrophoblast. However, over the ensuing days, the extraembryonic coelom separates the two structures and any splitting of the embryo in the second week will lead to reduplication of the amniotic membranes within the single chorionic enclosure. By the end of the second week, the amniotic cavity almost completely envelopes the developing embryo, and any division after this stage tends to be monoamniotic as well as monochorionic. The natural inci-

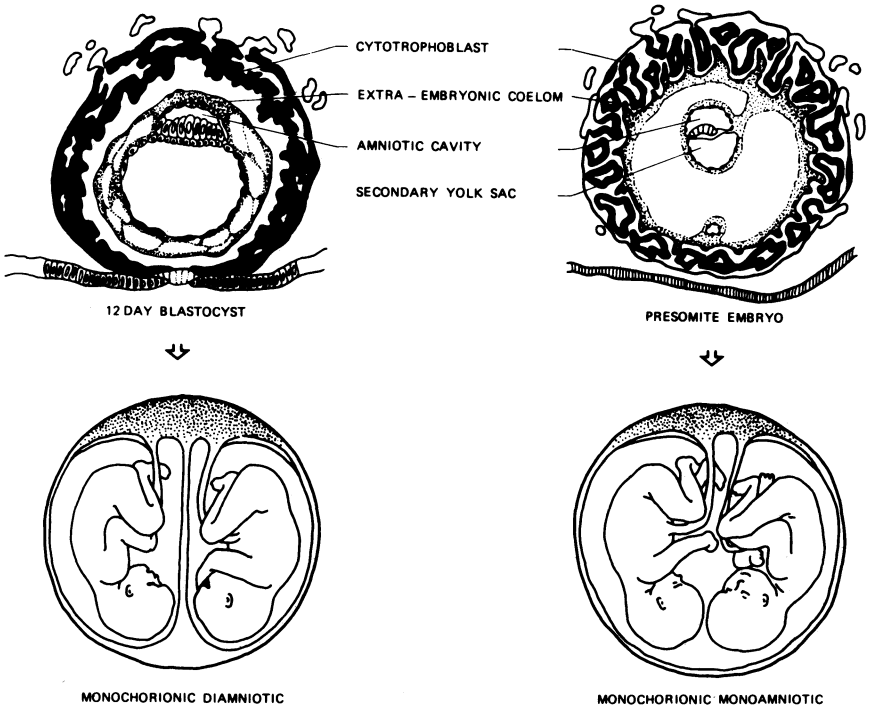


Figure 3
Division of the postimplantation stages of embryo development demonstrates greater sharing of trophoblast-derived tissue and amniotic sacs with later divisions. In the very late stage even embryonic germ layer derivatives may be shared, leading to conjoined twins. (Modified from Hertig et al.[10])

dence of this latter phenomenon is around 1 in 8000.[9] Incomplete separations at this stage can lead to the very rare event of conjoined twins. We know of 12 sets of twins that have been delivered following IVF-ET. This is a high incidence in ~ 200 IVF deliveries. These have invariably been dizygotic twins arising from multiple ETs. The majority of multiple transfers have in fact led to singleton pregnancies, although the overall chance of pregnancy improves proportionately with the number of embryos transferred. This suggests that a minority of the embryos generated from IVF have the potential to implant and establish an ongoing pregnancy. When pregnancy was established in this case, it was assumed that both embryos had been successfully implanted. However, the evidence is overwhelmingly in favor of one embryo implanting with subsequent separation and reduplication somewhere between days 7 and 14 following fertilization. Because of the rare possibility of fusion of the intervening membranes with disappearance of the chorionic layers, additional markers were assessed to confirm the hypothesis of monozygosity.

We have carefully checked the appearance of the infants, with particular attention to minute details, including dermatoglyphic patterns (P = 0.26 for dizygosity), and compared their ABO blood grouping and Rhesus type with an assessment of the genotype. In addition, five other blood groupings have been compared and found to be identical (P = 0.05 for dizygosity). The HLA family study shows that the twins are haploidentical for the locus A and B alleles (P = 0.25 for dizygosity). Similarly, banding techniques in the family study revealed a marker chromosome on maternal 9, which did not appear in either twin, and their banding patterns were identical (P = 0.25 for dizygosity). Each of the marker studies, although insensitive in their own right for confirming monozygosity, do support the hypothesis arising from the placental and membrane configuration and the clinical appearance of the twins, that they are derived from only one of the two embryos which were transferred. Together, the marker studies reveal the chance for dizygosity as $P <$ 0.001.

The possibility of the IVF technique or manipulations undertaken on the embryos prior to transfer, causing embryo splitting, was considered. Two manipulative techniques were used in our system, one of fine needle dissection and the other of fine pipetting, to divest the embryo of cumulus and coronal cells. However, these maneuvers are undertaken prior to the 4-cell stage, when dichorionic, diamniotic twins would be expected. Nevertheless, the intervening chorion between twins may fuse and disappear to convert a dichorionic placenta into an apparent monochorionic arrangement. It is suggested, therefore, that twins arising from IVF be carefully examined for this possibility. It is more likely, however, that the twins described arose because of a natural event which occurred after zona hatching, during the very early implantation phase. This is not likely to bear any relationship to their mode of conception.

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